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(54) Title: PRODUCTION OF FOREIGN POLYPEPTIDES IN PLANTS AS VIRAL COAT PROTEIN FUSIONS

(57) Abstract: The present invention relates to foreign peptide sequences fused to the amino-terminal of plant viral structural proteins and a method of their production. Fusion proteins are economically synthesized in plants at high levels by biologically contained tobamoviruses. The foreign peptide sequences can be cleaved from the fusion proteins by proteolytic enzymes or chemical reagents. The foreign peptide sequences of the invention have many uses. Such uses include use as antigens for inducing the production of antibodies having desired binding properties, e.g., protective antibodies, for use as vaccine antigens for the induction of protective immunity, including immunity against parasitic infections, for use as a protein involved in hormonal activity, or for use as a protein involved in immunoregulatory activity.

PRODUCTION OF FOREIGN POLYPEPTIDES IN PLANTS AS VIRAL COAT PROTEIN FUSIONS

FIELD OF THE INVENTION

The present invention in the field of genetically engineered polypeptide production in plants, is directed more specifically to the use of tobamovirus vectors to express fusion proteins such as immunogenic proteins useful as vaccine compositions in humans and other mammals.

BACKGROUND OF THE INVENTION

Polypeptides (used interchangeably with peptides) are an important class of biomolecules that are active in their natural setting or can be harnessed as pharmacological agents, to act as hormones, cytokines, vaccine antigens, immunoregulatory agents, enzyme inhibitors, cell adhesion molecules, receptor domains and the like. The cost of chemical synthesis limits the utility of synthetic peptides as therapeutic drugs or vaccines. There exists a need for rapid, inexpensive synthesis of milligram and greater quantities of naturally-occurring polypeptides. Animal and bacterial viruses have been harnessed successfully as "carriers" of, or as a source of, recombinant nucleic acids that encode and are made to express desired polypeptides.

Plants, which are safe and inexpensive to grow, represent improved alternative hosts for cost-effective production of such peptides. There is need in the art for new and improved vectors for the genetic manipulation of plants. Nevertheless, during the last decade, considerable progress has been made in expressing heterologous (foreign) genes in plants. Heterologous proteins are now routinely produced in many plant species for purposes of improving the plant or for protein production *per se*. Animal proteins have been successfully produced in plants (reviewed in Krebbers *et al.*, 1992). One advantage for use in human medicine is that proteins synthesized in plants are obtained relatively free of bacterial toxins and of other microorganisms or viruses that are pathogenic to humans.

Vectors for the genetic manipulation of plants have been derived from several naturally occurring plant viruses, including tobacco mosaic virus, TMV. TMV is the type member of the tobamovirus (TbmV) group. TMV exists as straight tubular virions of approximately 300 x 18 nm with a 4 nm-diameter hollow canal, consisting of approximately 2000 copies of a single capsid protein wound helically around a single RNA molecule. The composition of TMV virions is 95% protein and 5% RNA by weight. The TMV genome is a single-stranded RNA of 6395 nucleotides and includes five large open reading frames (ORFs). Expression of each ORF (or gene) is regulated independently. The virion RNA serves as the mRNA for the 5' ORFs encoding the 126 kDa

replicase subunit and the overlapping 183 kDa replicase subunit that is produced by read-through of an amber stop codon - a process that occurs at a frequency of about 5%. Expression of "internal" genes is controlled by different promoters on the minus-sense RNA strand that drive transcription of 3'-coterminal subgenomic mRNAs during replication (Figure 1). A detailed description of TbmV life cycle and gene expression is found in Dawson and Lehto, *Advances in Virus Research 38*:307-342 (1991).

For protein production in plants, viral vectors encoding heterologous genes that are transiently expressed have several advantages. Products of plant virus genes are among the most abundant proteins in virus-infected plants. Commonly, a viral gene product is the major protein produced in a plant cell during virus replication. Many viruses move rapidly from the initial infection site to almost all cells of the plant. For these reasons, plant viruses have been developed into efficient vectors for transient expression of heterologous proteins in plants. Viruses that infect multicellular plants are relatively small, probably due to the size limitation imposed by the pathways that permit virus movement from cell to cell in spreading a systemic infection to the entire plant. Most plant viruses have a single-stranded RNA genome of fewer than 10 kb.

Genetically altered plant viruses provide one efficient means for transducing plants with genes encoding fusion polypeptides ("FP") comprising a peptide or polypeptide of interest linked to a "carrier" sequence. For a description of TMV coat protein fusions, see Turpen *et al.*, U.S. Patent No. 5,977,438 ("Production of Peptides in Plants as Viral Coat Protein Fusions"). See also: Yusibov V. *et al.*, *Proc. Natl. Acad. Sci. USA 94*:5784-5788 (1997); Modelska, A *et al.*, *Proc. Natl. Acad. Sci. USA 95*:2481-2485 (1998).

PARVOVIRUS DISEASE

Parvovirus pathogenesis was recently reviewed by Parish, C.R., *Baillieres Clin. Haematol.* 8:57-71 (1995). Feline parvovirus (FPV) and canine parvovirus (CPV) are closely related as is pathophysiology of the diseases they cause. Parvoviruses replicate first in the tonsils, and then spread to its ultimate target cells: mitotically active intestinal crypt epithelial cells and bone marrow stem cells. Viremia lasts for less than 7 days followed either by recovery or death. Clinical signs in cats include fever, vomiting, diarrhea, panleukopenia, acute shock and can lead to death. The disease outcome and mortality is proportional to the severity of the leukopenia.

The mink enteritis virus (MEV) is closely related to FPV. The MEV VP2 (or E2) epitope was expressed on the surface of cowpea mosaic virus (CPMV) which was propagated on the leaves of the black-eyed bean (Dalsgaard, K *et al.*, *Nature Biotechnol. 15*:248-252 (1997)). One mg of the CPMV material displaying this epitope was used to immunize minks and protect them against

challenge with virulent MEV. The immunized minks were protected against clinical disease and shed very little virus when infected. The authors proposed that this epitope, expressed in this manner, could be used to protect cats and dogs against infection by their respective parvoviruses.

The coding sequence for VP2 (E2) and the rabies spike glycoprotein have been engineered into raccoon poxvirus to produce a live recombinant vaccine against rabies and feline panleukopenia (Hu, L. *et al.*, *Virology 218*:248-252 (1996); Hu, L. *et al.*, *Vaccine 15*: 1466-1472 (1997)). Cats vaccinated with this construct were protected against challenge with virulent parvovirus.

SUMMARY OF THE INVENTION

The present invention is directed to a polynucleotide (or nucleic acid) molecule that encodes a FP, which FP comprises a plant virus coat protein (VCP) and a polypeptide of interest (P1) at the N-terminus of the plant VCP via a linking element (L). Such a construct may be characterized as follows at the protein level:

H_2N--**P1--L--VCP**--COOH

A second polypeptide of interest ("P2") may be fused to the above fusion protein at the C-terminus of the plant VCP

H_2N-**P1--L--VCP--P2**-COOH

at the C-terminus of P1

H_2N-**P1--L--P2---L--VCP**-COOH

or at an internal site of the plant VCP

H_2N-**P1--L--VCP$_{(a)}$-P2-VCP$_{(b)}$**-COOH

wherein VCP(a) and VCP(b) refer to two fragments of VCP that flank the internally located P2 sequence.

Also included are FP's that are concatemers ccomprising between 3 and 100 peptides of interest, either identical or different, linked serially, with or without intervening L groups between each repeating peptide unit, ultimately linked to the VCP, preferably at its N-terminus.

By infecting plant cells with the recombinant plant viruses of the invention, relatively large quantities of the polypeptide of interest (P1 or P2) may be produced in the form of a FP.

P1 can be isolated from the plant VCP by chemical or enzymatic cleavage. A chemical cleaving agent breaks a covalent bond within L. The FP encoded by the recombinant plant virus may exist in any one of several forms (?)

The FP preferably retains one or more properties of P1. When P2 is present, the FP may also possess one or more properties of P2. The isolated P1 or P2 or the FP itself may be used as an immunogen for vaccinating a subject to induce a protective immune response which may comprise antibodies, reactive T lymphocytes (*e.g.*, cytotoxic T lymphocytes or CTL). A preferred P1 is a protective parvovirus epitope.

The present invention also encompasses methods for producing P1 or P2 by expressing the FP from the polynucleotide and cleaving P1 or P2 or both from the FP.

The invention provides a recombinant viral nucleic acid, a recombinant viral genome, recombinant virus particles, recombinant plant viruses, plants, plant cells, plant protoplasts, and the like, that comprise such polynucleotides. The invention also provides a polynucleotide that includes the genome of the subject recombinant plant virus.

The invention provides fusion polypeptides encoded by the subject recombinant plant viruses and plant cells that have been infected by the viruses.

The invention also provides for methods for synthesis of P1 by expressing the fusion polypeptide using the subject polynucleotide.

The present invention provides polynucleotides that encode FPs comprising a protein of interest linked to the N-terminus of a plant VCP via a linking element. The present invention also provides methods for the production of the protein of interest using the subject polynucleotides and FP. An advantage of having the protein of interest fused to the N-terminus of the plant VCP is that infectious virions are able to tolerate a higher proportion of VCP subunits with the FP than if the protein of interest were fused to the C-terminus or an internal site of the plant VCP. Other advantages of the invention are:

(1) the protein of interest is cleavable from the fusion protein in the presence of a cleaving agent.

(2) the fusion protein can be extracted using methods that are scalable

(3) subsequent cleavage of the protein of interest from the fusion protein is also scalable

(4) initiation of translation from an internal methionine codon results in the expression of wild-type plant VCP which results production of more virus particles, thereby facilitating virus isolation and purification.

DESCRIPTION OF THE FIGURES

Figure 1 depicts a Tobamovirus Gene Expression. The gene expression of tobamoviruses is diagrammed.

Figure 2 is a plasmid map of the TMV Transcription Vector pSNC004. The infectious RNA genome of the U1 strain of TMV is synthesized by T7 RNA polymerase in vitro from pSNC004 linearized with KpnI.

Figure 3 depicts a diagram of plasmid constructions. Each step in the construction of plasmid DNAs encoding various viral epitope fusion vectors discussed in the examples is diagrammed.

Figure 4 depicts the binding of monoclonal antibody (NVS3) to TMV291. The reactivity of NVS3 to the malaria epitope present in TMV291 is measured in a standard ELISA.

Figure 5 depicts the binding of monoclonal antibody (NYS1) to TMV261. The reactivity of NYS1 to the malaria epitope present in TMV261 is measured in a standard ELISA.

Figure 6 depicts the location of oligos and restriction sites used in the construction of pJL150/198 and pJL150/199.

Figure 7 depicts the Matrix-assisted laser desorption time of flight mass spectrometry (MALDI-TOF) analysis of PEG purified virion preparations derived from Supernatant 1 of Example 15 (see Table 6). The peaks showing the mass of three polypeptides were detected and corresponded to (1) the predicted full length TMV coat-fusion peptide (indicated by 19,163.8064), a proteolytic degradation product containing an N-terminal arginine residue (indicated by 18,104.8791), and a protein containing an N-terminal Met residue resulting from initiation of translation on an internal Met codon or proteolytic degradation (17,537.1190).

Figure 8 depicts the MALDI-TOF analysis of PEG purified virion preparations derived from Supernatant 2 of Example 15 (see Table 6). The peaks of three protein masses were detected corresponding to the predicted full length TMV coat-peptide fusion (indicated by 19,188.2016), (2) a proteolytic degradation product containing an N-terminal Arg residue (indicated by 18,121.6166), and (3) a polypeptide containing an N-terminal Met residue resulting either from translation that was initiated on an internal methionine codon or from proteolytic degradation (17,550.6212).

Figure 9 depicts the MALDI-TOF analysis of cyanogen bromide (CNBr) cleaved products in Example 16 (see Table 6). The CNBr pellet contained two products with mass weights of 19,330 and 17,570 Da corresponding to uncleaved and cleaved TMV coat protein, respectively. An apparent increase in mass is likely due to acid ester formation.

Figure 10 depicts the MALDI-TOF analysis of the resuspended permeate lyophilisate of Example 16 (see Table 6). The resuspended 10 kDa permeate lyophilisate sample contained predominantly a 1736 Da species and minor quantities of 1720, 1758 and 1828 Da species. The

1736 fragment corresponds to the predicted mass of the released parvovirus peptide sequence containing a C-terminal homoserine.

Figure 11 depicts the MALDI-TOF analysis of the resuspended permeate lyophilisate of Example 16 (see Table 6). The resuspended 10 kDa permeate lyophilisate sample did not contain detectable amounts of uncleaved or cleaved TMV coat.

Figure 12 depicts the HPLC chromatograms of Pellet 1 of Example 19 (see Table 6).

Figure 13 depicts the HPLC chromatograms of Supernatant 1 of Example 19 (see Table 6).

Figure 14 depicts the mass spectrometric data for construct 149 grown in *Nicotiana benthamiana* and purified by pH and heat treatment.

Figure 15 depicts the mass spectrometric data for construct 149 grown in *Nicotiana tabacum* and purified by pH and heat treatment.

Figure 16 depicts the mass spectrometric data for construct 150 grown in *Nicotiana benthamiana* and purified by PEI treatment method.

Figure 17 depicts the mass spectrometric data for construct 150 grown in *Nicotiana benthamiana* and purified by pH and heat treatment.

Figure 18 depicts the mass spectrometric data for construct 149 grown in *Nicotiana tabacum* and purified by pH and heat treatment.

Figure 19 depicts the mass spectrometric data for construct 150 grown in *Nicotiana tabacum* and purified by pH and heat treatment.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

A number of abbreviations and definitions used herein are as follows:

CNBr: cyanogen bromide.

Coding Sequence: A DNA or RNA sequence which, when either transcribed and translated or directly translated, resulting in the formation of a polypeptide or a ribonucleotide sequence which, when translated, results in the formation of a polypeptide.

CP: Coat protein.

ELISA: Enzyme-linked immunosorbent assay.

FP: Fusion polypeptide (or protein)

FPV: Feline panleukopenia virus.

HPLC: High Performance Liquid Chromatography

Infection: The ability of a virus to transfer its nucleic acid to a host or introduce a viral nucleic acid into a host, wherein the viral nucleic acid is replicated, viral proteins are synthesized, and new viral particles assembled. In this context, the terms

"transmissible" and "infective" are used interchangeably herein. The term is also meant to include the ability of a selected nucleic acid sequence to integrate into a genome, chromosome or gene of a target organism.

MALDI-TOF: Matrix-assisted laser desorption time of flight mass spectrometry.

Nucleic acid: any DNA or RNA molecule consisting of one or more nucleotides up to and including a complete gene sequence. The term is intended to encompass all nucleic acids whether naturally occurring (native) or engineered or recombinant.

ORF: Open reading frame.

PAGE: Polyacrylamide gel electrophoresis.

PCR: Polymerase chain reaction.

PEG: Polyethylene glycol.

PEI: Polyethyleneimine.

Protoplast: An isolated plant or bacterial cell lacking some or all of its cell wall.

Recombinant Viral Nucleic Acid: Viral nucleic acid which has been modified to contain non-native nucleotide sequences which may be derived from any organism or may be purely synthetic; however, such nucleic acids include sequences naturally occurring in the organism into which the recombinant viral nucleic acid is to be introduced.

Recombinant Virus: A virus containing the recombinant viral nucleic acid.

Subgenomic Promoter: A promoter of a subgenomic mRNA transcribed from a viral nucleic acid.

Systemic Infection: infection throughout a substantial part of an organism including mechanisms of spread other than mere direct cell inoculation but rather including transport from one infected cell to additional cells either nearby or distant.

TbmV: Tobamovirus

TMV: Tobacco mosaic virus.

VCP: Viral coat protein.

Viral Particle: High molecular weight aggregate of viral structural proteins with or without genomic nucleic acid

Virion: An infectious viral particle.

For ease of expression, the following formulas are used herein:

P1: polypeptide of interest

L: linking element or linker (*e.g.*, between P1 and VCP and/or P2 and VCP and/or P1 and P2. Different L's are designated L^1, L^2, *etc.*

P2: a second polypeptide of interest that differs from P1.

Thus, one example of a FP is P1--L-VCP.

The Invention

The invention provides novel recombinant plant viruses that encode fusion polypeptides that combine a plant VCP and a polypeptide of interest preferably fused to the N-terminus of the plant VCP via a linker. Recombinant plant viruses of the invention have the property of systemically expressing the fusion polypeptide, by infecting plant cells and then spreading to render the infection systemic. The invention provides methods for isolating the polypeptide of interest from the fusion polypeptide by the cleaving the linker using a cleaving agent. Thus, large quantities of a polypeptide of interest, either fused to the plant VCP or isolated from the fusion polypeptide, are produced using the recombinant plant viruses of the invention.

The fusion polypeptides of the invention include at least three components: (1) a plant VCP, (2) a linker, and (3) a polypeptide of interest (P1). The fusion polypeptide may also include a second polypeptide of interest. The plant VCP component may be derived from a native CP of the plant virus from which the expression vector is primarily derived,. That is, such a VCP is native or homologous with respect to the recombinant viral vector genome. Alternatively, the VCP component of the invention may be heterologous (non-native) to the recombinant viral vector genome. The plant VCP component is not necessarily the wild-type VCP or a naturally occurring plant VCP at all. However, such a plant VCP component preferably exhibits the relevant biological/chemical characteristics of the wild-type or naturally occurring plant VCPs that permit it to function in accordance with the invention. The invention also contemplates, however, modified VCPs wherein the P1 or P2 or other peptide fusion partner is of such magnitude or is situated in a way that interferes with biological function of the VCP. This is because other means, well-known in the art, are available for purification of the desired peptide; moreover, virion assembly is not necessary for systemic infection of the plant.

In a preferred embodiment, the polynucleotide encodes a fusion polypeptide expressed with the plant VCP. The expression of the plant VCP may begin at the internal initiation codon of the plant VCP DNA (or mRNA) during translation or post-translation modification of the fusion polypeptide. The proteolysis of the fusion polypeptide is a post-translational modification that gives rise to the plant VCP.

In a preferred embodiment of the invention, the VCP is the TMV 17.5 kDa CP and is used with a TMV derived vector.

The linker may be any covalent bond or peptide of virtually any amino acid sequence that is cleaved by a cleaving agent that breaks this covalent bond or any peptide bond. This separates the plant VCP from the polypeptide of interest.

The cleaving agent may be a chemical compound or a protease capable of breaking the relevant covalent bond. Examples of such proteases are trypsin, chymotrypsin, pepsin, *Staphylococcus aureus* V8 protease, and Factor Xa protease. An example of a chemical compound is cyanogen bromide ("CNBr").

In a preferred embodiment of the invention, a linker is one or more amino acids or a peptide that is susceptible to cleavage by a specific cleaving agent as described herein.

Example of cleaving agents and properties of their substrate linkers are shown in the table below.

CLEAVING AGENT	TARGET LINKERS
Trypsin	Lys, Arg
Chymotrypsin	Phe, Trp, Tyr
Pepsin	Phe, Trp, Tyr, Leu, Asp, Glu
S. aureus V8 protease[1]	Asp, Glu
Factor Xa protease	Ile-Glu-Gly-Arg (peptide)
CNBr	Met

[1] *S. aureus* V8 protease cleaves at the carboxylic side of glutamate in 50 mM ammonium bicarbonate (pH 7.8), or 50 mM ammonium acetate (pH 4.0); and at the carboxylic side of both aspartate or °glutamate in 50 mM sodium phosphate buffer (pH 7.8)).

The foregoing examples merely illustrate but do not limit the possible cleaving agent and linking element combinations. In a preferred embodiment, one covalent bond broken is a peptide bond.

The polypeptide of interest in the fusion polypeptide may consist of a peptide of virtually any amino acid sequence, provided that it maintains the relevant biological/biochemical activity examples of which are (1) binding to a receptor molecule, including an antibody or T cell receptor (2) binding to the active site of an enzyme (3) immunogenicity or immunoregulatory activity (4) hormonal activity, and (6) metal chelating activity. The polypeptide of interest may include all or part of the amino acid sequence of the protein from which it is derived, *e.g.*, an antigenic epitope. For example, for use in a Hepatitis B vaccine, a partial amino acid sequence that has antigenic properties similar to hepatitis B surface antigen (HBSAg) may be the polypeptide of interest within a fusion polypeptide invention. Detailed structural and functional information about any protein is used in the design of a fusion polypeptide having the desired properties of the polypeptide of interest.

As defined herein, the polypeptide of interest may vary in size from one amino acid residue to over several hundred residues. Preferably the polypeptide of interest has a length of < 100 amino acid residues, more preferably, < 50 residues, even more preferably, < 25 residues. It will be appreciated by those of ordinary skill in the art that, in some embodiments, the polypeptide of interest portion may need to be longer > 100 residues in order to maintain the desired properties. In general, it is preferred to minimize the length of the polypeptide of interest component of the present fusion while retaining the desired biological/chemical properties.

Particularly preferred embodiments are polypeptides or peptides for use as immunogens and vaccine compositions. For example, the fusion polypeptide or an immunogenic fragment thereof is injected into a mammal, optionally along with a suitable adjuvant; to induce an immune response directed against P1. P1 may include one or more epitopes of a pathogenic microorganism such that the immune response results in a state of protective immunity in the immunized host. The same immune response can be exploited for producing antisera or monoclonal antibodies that are used as immunoassay reagents.

The FP of the invention may also have a second polypeptide of interest P2 that differs from P1 and is linked to the VCP at a position other than its N-terminus. The site in the FP where the VCP sequence is joined to the P2 sequence is referred to herein as the "fusion joint." A given FP may have one (or two) fusion joints located at the C-terminus of the VCP that joins the N-terminus of P2. In other embodiments, the P2 sequence is internal to the VCP sequence so that the FP has two fusion joints at either terminus of P2. This is termed an internal FP. An internal FP may comprise a full length or partial VCP sequence that is "interrupted" by the P2 sequence. This structure can be described as

$$H_2N\text{-}VCP_{(a)}\text{-}CO\text{-}NH\text{-}P2\text{-}CO\text{-}NH\ VCP_{(b)}COOH$$

Here, $VCP_{(a)}$ and $VCP_{(b)}$ refer to two segments of VCP, wherein $VCP_{(a)}$ is N-terminal to the intervening P2 and the $VCP_{(b)}$ is C-terminal to P2. The fusion joints are shown as peptide bonds (CO-NH) but can include a linking element at either or both of these joints. If two linking elements are present, they can be the same or different. A formula representing the above FP with two different linking elements L^1 and L^2 is shown as follows:

$$H_2N\text{-}VCP_{(a)}\text{-}L^1\text{-}P2\text{-}L^2\text{-}VCP_{(b)}\text{-}COOH$$

In an internal FP, the fusion joints may be located at any of a number of sites within the VCP. Suitable sites for the fusion joints may be determined through routine systematic variation of the position and testing of the product for the desired properties. Suitable sites may be determined by prior analysis of the three dimensional structure of the VCP to determine which sites permit

insertion of P2 without significantly interfering with VCP function. Detailed three dimensional structures of plant VCPs and their orientation in the virus are known and publicly available. For example, a resolution model of the virus and the CP of Cucumber Green Mottle Mosaic Virus (which bears strong structural similarities to other tobamovirus CPs) are found in Wang and Stubbs, *J. Mol. Biol.* 239:371-384 (1994). Detailed structural information on TMV and its CP are found in Namba *et al.*, *J. Mol. Biol.* 208:307-325 (1989) and Pattanayek and Stubbs *J. Mol. Biol.* 228:516-528 (1992).

Other peptides useful in the present invention, particularly as a second peptide of interest (*i.e.*, P2) are ones that serve as affinity tags that permit purification of the FP. A well known example is a "His-Tag" wherein a sequence of 5-7 histidine residues comprise P2 and permit affinity chromatography with a commercially available Nickel-Agarose columns (Qiagen) for purification of the His tagged protein. Other tags that can be included in the present FPs are the maltose binding protein, the cellulose-biding protein or domains or fragments thereof having binding activity. Affinity purification then utilizes maltose or cellulose matrices or columns. c-myc is another useful tag. Finally, any antigenic epitope to which an antibody, preferably a monoclonal antibody is available, can serve as an affinity tag for use with the immobilized antibody. Therefore, virtually any peptide that is antigenic can serve in this capacity as P2 to assist in purification of a P1-containing FP in which the particular P2 is a component.

One formula representing such a preferred His-tagged FP is shown by **P1-L-His_6-VCP**, wherein the linker L is Met, cleavable by cyanogen bromide.

Knowledge of the three dimensional structure of a plant virus particle and its assembly process permits the design of various VCP fusion polypeptides that may include insertions and partial substitutions. For example, if P1 is hydrophilic, it may be appropriate to fuse it to a surface loop region of the TMV VCP. Likewise, α-helical segments that maintain subunit contacts might be substituted for appropriate regions of the TMV VCP α helices or nucleic acid binding domains expressed in the region of the VCP oriented towards the RNA.

Polynucleotide sequences encoding the present fusion polypeptides may comprise a "leaky" stop codon at a fusion joint. A leaky stop codon is one that does not always result in translation termination but rather is periodically translated. The leaky stop codon may be immediately adjacent to the fusion joint, or may be located near (*e.g.*, within 9 bases) the fusion joint. Use of a leaky stop codon maintains a desired ratio of FP to wild-type VCP. The frequency of initiation or

termination at a given start or stop codon is "context dependent." Ribosomes "scan" from the 5'-end of a mRNA for the first ATG codon. In a non-optimal sequence context, the ribosome will pass this codon at some frequency and continue moving to the next available start codon and initiate translation from that downstream position. Similarly, the first termination codon encountered during translation will not be recognized with absolute efficiency if it is in a particular sequence context. Consequently, many naturally occurring proteins exist as a heterogeneous population of polypeptides having heterogeneous N and/or C terminal extensions.

Thus, a leaky stop codon at a fusion joint coding region of an FP-encoding recombinant viral vector, may be exploited to produce both a FP and a second smaller polypeptide, *e.g.*, the VCP. A leaky stop codon may be used at, or proximal to, the fusion joints of FPs in which P2 is joined to the C-terminus of the VCP. The single recombinant viral vector would then reduce both the FP and the VCP. Moreover, a leaky start codon may be used at or proximal to the fusion joints of am FP in which P1 or P2 is joined to the N-terminus of the VCP, resulting in a similar outcome.

In the case of TMV VCP, extensions at the N and C termini end up at the surface of virus particles projecting away from the helical axis.

An example of a leaky stop sequence occurring at the junction of the 126/183 kDa reading frames of TMV was described in 1978 (Pelham, 1978). Skuzeski *et al.* (1991) defined the 3' context requirement of this region as CAR-YYA (C=cytosine, A=adenine, R=purine, Y=pyrimidine) which conferred leakiness of termination on a heterologous protein marker gene (β-glucuronidase).

In another embodiment of the invention, the fusion joints are designed as amino acid sequences that serve as substrates for a protease. A FP having such a fusion joint permits a convenient means for deriving P1 or P2 from the FP using a suitable proteolytic enzyme that is effective *in vitro* or *in vivo*.

Expression of the present FP may be driven by any of a variety of promoters that are operative with the nucleic acid of the recombinant plant virus. Promoter should be functional in a plant cell or plant. The promoter is preferably placed 5' to the FP coding sequence.

In a preferred embodiment, the FP is expressed in a plant cell (whether grown in culture or part of a plant) or a plant protoplast.

The promoter may be any viral promoter, preferably an RNA viral promoter, more preferably, a promoter of a single-stranded plus-sense RNA virus. In a more preferred embodiment, the promoter is a tobamovirus promoter, most preferably, a TMV promoter. An example of a preferred TMV promoter is the promoter of the TMV VCP gene.

The FP is preferably expressed from a plant virus subgenomic promoter using vectors such as those as described in U.S. Patent 5,316,931.

Expression levels may be elevated or controlled by a variety of plant or viral transcription factors.

In one embodiment, the FP has an internal Met, or another amino acid capable of initiating translation, near the N-terminus of the FP that serves as the initiation point of translation. Initiation of translation from such an internal Met (or other amino acid) results in the expression of a wild-type VCP or peptide thereof that yields a higher number of viral particles then in the absence of such internal initiation of the VCP (or peptide).

In one embodiment, the internal Met (or other amino acid) is part of the linking element of the FP.

Preferably, the internal Met (or other amino acid) is situated less than 100 residues away from the N-terminal residue of the FP, more preferably, less than 50 acids away, more preferably less than 25 residues away, even more preferably less than 20 amino acids away. It is preferred that the internal Met be cleavable by CNBr.

Recombinant DNA technology allows artificial extension of the life cycle of numerous plant RNA viruses through a DNA stage that facilitates manipulation of the viral genome. These techniques are useful for making the recombinant plant viruses of the present invention. A cDNA of the entire TMV genome has been cloned and functionally linked to a bacterial promoter in an *E. coli* plasmid (Dawson *et al.*, 1986). Infectious recombinant plant viral RNA transcripts may also be produced using other conventional techniques, for example, with the commercially available RNA polymerases from phages T7, T3 or SP6. Precise replicas of the virion RNA can be produced *in vitro* using RNA polymerase and dinucleotide cap, m7GpppG. This allows manipulation of the viral genome including making the virus into a vector for expressing foreign genes. A method of producing plant RNA virus vectors based on manipulating RNA fragments with RNA ligase proved to be impractical and is not widely used (Pelcher, 1982).

Details for making and using recombinant RNA plant viruses are found, in U.S. Patent 5,316,931 (Donson *et al.*), which is herein incorporated by reference.

The present invention provides a polynucleotide encoding a recombinant RNA plant vector for the expression of the FP of the invention.

The invention also provides polynucleotides comprising a portion or portions of the subject vectors. The vectors described in U.S. Patent 5,316,931 (see above) are particularly suited for expressing the FPs

Also provided are virus particles that comprise the polynucleotide molecules of the invention. The viral coat may consist entirely of FP encoded by the polynucleotide of the invention, or of a mixture of FPs and non-fused VCPs, wherein the ratio of the two polypeptides may be varied. As tobamovirus coat proteins may self-assemble into virus particles, the virus particles of the invention may be assembled either *in vivo* or *in vitro*. The virus particles may be conveniently disassembled using well known techniques to simplify the purification of the FPs, or fragments thereof.

The invention also provides for recombinant plant cells, plant cells, plant protoplasts, and the like comprising the subject fusion polypeptides and/or virus particles comprising the subject fusion polypeptides. These cells may be produced by infecting cells (either in culture or in whole plants) with infections virus particles of the invention or by transfecting with polynucleotides that are or that encode the genome of the infectious virus particles. These cells have many uses, for example, primarily as a source or site for expressed FPs.

P1 or P2 may comprise many different amino acid residue sequences, and accordingly, may possess many different possible biological/chemical properties. In a preferred embodiment, the P1 or P2 component of the FP is useful as a vaccine composition. The surface of TMV particles and other tobamoviruses include highly antigenic continuous epitopes with segmental mobility, thereby making TMV particles especially useful in producing a desired immune response. These properties make the virus particles of the invention especially useful as carriers of foreign epitopes for presentation to the mammalian immune system.

While the recombinant RNA viruses of the invention may be used to produce a variety of FPs use as vaccine compositions or precursors thereof, malaria vaccines are of particular interest. Human malaria, caused by the protozoan species *Plasmodium falciparum, P. vivax, P. ovale* and *P. malariae*, is transmitted by the sporozoite form spread by the Anopheles mosquito. Control of malaria will likely require safe and stable vaccines. Several peptide epitopes expressed during various stages of the parasite life cycle are thought to contribute to the induction of protective immunity in partially resistant individuals living in endemic areas and in individuals experimentally immunized with irradiated sporozoites.

When the FPs of the invention, fragments thereof, or viral particles comprising the FPs are used *in vivo*, the polypeptides are typically administered in a composition comprising a pharmaceutical carrier. A pharmaceutical carrier can be any compatible, non-toxic substance suitable for delivery of these agents to the body. Sterile water, alcohol, fats, waxes and inert solids may be included in the carrier. Pharmaceutically accepted adjuvants (buffering agents, dispersing

agent) may also be incorporated into the pharmaceutical composition. Additionally, when the polypeptide of interest, the FP, or the fragment thereof, is to be used to induce an immune response (protective or otherwise), the formulation may comprise one or more immunological adjuvants.

The pharmaceutical compositions of the invention comprising P1, P2, the FP, or a fragment thereof, may be administered to a subject, human or animal, by a variety of routes, including: oral or parenteral, *i.e.,* subcutaneously, intra-dermal, intra-muscular, intravenous or other route of injection or infusion. Thus, compositions for parenteral administration comprise a solution of P1, P2 or the FP, or a fragment or derivative thereof, or a "cocktail" of one or more of the foregoing, dissolved or dispersed in an acceptable, preferably aqueous, carrier such as water, buffered water, 0.4% saline, 0.3% glycerin and the like. These solutions are preferably sterile and free of particulate matter. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH-adjusting and buffering agents and salts, toxicity-diminishing agents and the like, for example sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate, *etc.* The concentration of the polypeptide or protein in these formulations can vary widely depending on the specific polypeptide and desired biological activity, *e.g.*, from less than about 0.5%, usually at or at least about 1% to as much as 15 or 20% by weight and will be selected primarily based on fluid volumes, viscosity's, etc., in accordance with the intended mode and route of administration.

Actual methods for preparing parentally administrable compositions and adjustments necessary for administration to subjects will be known or apparent to those skilled in the art and are described in more detail in, for example, *Remington's Pharmaceutical Science*, current edition, Mack Publishing Company, Easton, Pa, which is incorporated herein by reference.

The invention also encompasses methods for producing P1 or P2 or FP by expressing the FP-encoding nucleic acid molecule. The method comprises contacting a plant or a plant cell with a recombinant plant viral nucleic acid that includes the subject coding sequence; and growing, expanding or cultivating the plant or the plant cell under conditions favorable for expression of the FP. The method may further comprise subjecting the FP to a cleaving agent active on the linking element either *in vitro* or *in vivo* such that one or more covalent bonds between P1 or P2 and the plant VCP is broken. The covalent bond to be broken is preferably a peptide bond.

The method may further comprise isolating or purifying the FP or isolating and/or purifying P1 or P2 from the plant VCP. The polypeptide of interest may be separated from the plant VCP by mechanical means, such as by centrifugation or ultrafiltration, or by HPLC. The method may

further comprise isolating recombinant viral particles comprising the subject polynucleotide or the subject fusion polypeptide from the rest of the plant or plant cell.

The following examples further illustrate the present invention. These examples are intended merely to be illustrative of the present invention and are not to be construed as being limiting.

EXAMPLES

Biological Deposits

The following present examples are based on a full length insert of wild type TMV (U1 strain) cloned in the vector pUC18 with a T7 promoter sequence at the 5'-end and a KpnI site at the 3'-end (pSNC004, Figure 2) or a similar plasmid pTMV304. Using the PCR technique and primers WD29 (SEQ ID NO: 1) and D1094 (SEQ ID NO: 2) (See Table 1 for nucleotide and amino acid sequences cited in Examples 1-4.), a 277 XmaI/HindIII amplification product was inserted with the 6140 bp XmaI/KpnI fragment from pTMV304 between the KpnI and HindIII sites of the common cloning vector pUC18 to create pSNC004. The plasmid pTMV304 is available from the American Type Culture Collection, Rockville, Maryland (ATCC accession no. 45138). The genome of the wild type TMV strain can be synthesized from pTMV304 using the SP6 polymerase, or from pSNC004 using the T7 polymerase. The wild-type TMV strain can also be obtained from the American Type Culture Collection, Rockville, Maryland (ATCC accession no. PV135). The plasmid pBGC152, Kumagai, M., *et al.*, (1993), is a derivative of pTMV304 and is used only as a cloning intermediate in the examples described below. The construction of each plasmid vector described in the examples below is diagrammed in Figure 3.

Table 1. Nucleotide and amino acid sequences cited in Examples 1-4.

SEQ ID NO: 1: ggaattcaag cttaatacga ctcactatag tatttttaca acaattacc
SEQ ID NO: 2: ccttcatgta aacctctc
SEQ ID NO: 4: taatcgatga tgattcggag gctac
SEQ ID NO: 5: aaagtctctg tctcctgcag ggaacctaac agttac
SEQ ID NO: 6: attatgcatc ttgactacct aggttgcagg accaga
SEQ ID NO: 7: ggcgatcggg ctggtgaccg tgca
SEQ ID NO: 8: cggtcaccag cccgatcgcc tgca
SEQ ID NO: 9: atg tct tac agt atc act act cca tct cag ttc gtg ttc ttg tca tca Met Ser Tyr Ser Ile Thr Thr Pro Ser Gln Phe Val Phe Leu Ser Ser 1 5 10 15 GCG TGG GCC GAC CCA ATA GAG TTA ATT AAT TTA TGT ACT AAT GCC TTA Ala Trp Ala Asp Pro Ile Glu Leu Ile Asn Leu Cys Thr Asn Ala Leu 20 25 30 GGA AAT CAG TTT CAA ACA CAA CAA GCT CGA ACT GTC GTT CAA AGA CAA Gly Asn Gln Phe Gln Thr Gln Gln Ala Arg Thr Val Val Gln Arg Gln

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        35                  40                  45

TTC AGT GAG GTG TGG AAA CCT TCA CCA CAA GTA ACT GTT AGG TTC CCT
Phe Ser Glu Val Trp Lys Pro Ser Pro Gln Val Thr Val Arg Phe Pro
    50                  55                  60

GCA GGC GAT CGG GCT GGT GAC CGT GCA GGA GAC AGA GAC TTT AAG GTG
Ala Gly Asp Arg Ala Gly Asp Arg Ala Gly Asp Arg Asp Phe Lys Val
65                  70                  75                  80

TAC AGG TAC AAT GCG GTA TTA GAC CCG CTA GTC ACA GCA CTG TTA GGT
Tyr Arg Tyr Asn Ala Val Leu Asp Pro Leu Val Thr Ala Leu Leu Gly
                85                  90                  95

GCA TTC GAC ACT AGA AAT AGA ATA ATA GAA GTT GAA AAT CAG GCG AAC
Ala Phe Asp Thr Arg Asn Arg Ile Ile Glu Val Glu Asn Gln Ala Asn
            100                 105                 110

CCC ACG ACT GCC GAA ACG TTA GAT GCT ACT CGT AGA GTA GAC GAC GCA
Pro Thr Thr Ala Glu Thr Leu Asp Ala Thr Arg Arg Val Asp Asp Ala
        115                 120                 125

ACG GTG GCC ATA AGG AGC GCG ATA AAT AAT TTA ATA GTA GAA TTG ATC
Thr Val Ala Ile Arg Ser Ala Ile Asn Asn Leu Ile Val Glu Leu Ile
    130                 135                 140

AGA GGA ACC GGA TCT TAT AAT CGG AGC TCT TTC GAG AGC TCT TCT GGT
Arg Gly Thr Gly Ser Tyr Asn Arg Ser Ser Phe Glu Ser Ser Ser Gly
145                 150                 155                 160

TTG GTT TGG ACC TCT GGT CCT GCA ACT TGA SEQ ID NO:10:
Leu Val Trp Thr Ser Gly Pro Ala Thr
                165                 169
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```
SEQ ID NO:10:
Met Ser Tyr Ser Ile Thr Thr Pro Ser Gln Phe Val Phe Leu Ser Ser
  1                 5                  10                  15

Ala Trp Ala Asp Pro Ile Glu Leu Ile Asn Leu Cys Thr Asn Ala Leu
            20                  25                  30

Gly Asn Gln Phe Gln Thr Gln Gln Ala Arg Thr Val Val Gln Arg Gln
        35                  40                  45

Phe Ser Glu Val Trp Lys Pro Ser Pro Gln Val Thr Val Arg Phe Pro
    50                  55                  60

Ala Gly Asp Arg Ala Gly Asp Arg Ala Gly Asp Arg Asp Phe Lys Val
65                  70                  75                  80

Tyr Arg Tyr Asn Ala Val Leu Asp Pro Leu Val Thr Ala Leu Leu Gly
                85                  90                  95

Ala Phe Asp Thr Arg Asn Arg Ile Ile Glu Val Glu Asn Gln Ala Asn
            100                 105                 110

Pro Thr Thr Ala Glu Thr Leu Asp Ala Thr Arg Arg Val Asp Asp Ala
        115                 120                 125
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Thr Val Ala Ile Arg Ser Ala Ile Asn Asn Leu Ile Val Glu Leu Ile
    130                 135                 140

Arg Gly Thr Gly Ser Tyr Asn Arg Ser Ser Phe Glu Ser Ser Ser Gly
145                 150                 155                 160

Leu Val Trp Thr Ser Gly Pro Ala Thr
                165             169
```

SEQ ID NO: 12: ctagcaatta caaggtccag gtgcacctca aggtcctgga gctcc

SEQ ID NO: 13: ctagggagct ccaggacctt gaggtgcacc tggaccttgt aattg

SEQ ID NO: 15:

```
Met Ser Tyr Ser Ile Thr Thr Pro Ser Gln Phe Val Phe Leu Ser Ser
  1               5                  10                  15

Ala Trp Ala Asp Pro Ile Glu Leu Ile Asn Leu Cys Thr Asn Ala Leu
            20                  25                  30

Gly Asn Gln Phe Gln Thr Gln Gln Ala Arg Thr Val Val Gln Arg Gln
        35                  40                  45

Phe Ser Glu Val Trp Lys Pro Ser Pro Gln Val Thr Val Arg Phe Pro
    50                  55                  60

Asp Ser Asp Phe Lys Val Tyr Arg Tyr Asn Ala Val Leu Asp Pro Leu
65                  70                  75                  80

Val Thr Ala Leu Leu Gly Ala Phe Asp Thr Arg Asn Arg Ile Ile Glu
                85                  90                  95

Val Glu Asn Gln Ala Asn Pro Thr Thr Ala Glu Thr Leu Asp Ala Thr
            100                 105                 110

Arg Arg Val Asp Asp Ala Thr Val Ala Ile Arg Ser Ala Ile Asn Asn
        115                 120                 125

Leu Ile Val Glu Leu Ile Arg Gly Thr Gly Ser Tyr Asn Arg Ser Ser
    130                 135                 140

Phe Glu Ser Ser Ser Gly Leu Val Trp Thr Ser Gly Pro Ala Thr Tyr
145                 150                 155                 160

Gln Leu Gln Gly Pro Gly Ala Pro Gln Gly Pro Gly Ala Pro
                165                 170             174
```

SEQ ID NO:16:

```
ATG TCT TAC AGT ATC ACT ACT CCA TCT CAG TTC GTG TTC TTG TCA TCA
Met Ser Tyr Ser Ile Thr Thr Pro Ser Gln Phe Val Phe Leu Ser Ser
  1               5                  10                  15

GCG TGG GCC GAC CCA ATA GAG TTA ATT AAT TTA TGT ACT AAT GCC TTA
Ala Trp Ala Asp Pro Ile Glu Leu Ile Asn Leu Cys Thr Asn Ala Leu
            20                  25                  30

GGA AAT CAG TTT CAA ACA CAA CAA GCT CGA ACT GTC GTT CAA AGA CAA
```

```
Gly Asn Gln Phe Gln Thr Gln Gln Ala Arg Thr Val Val Gln Arg Gln
          35                  40                  45

TTC AGT GAG GTG TGG AAA CCT TCA CCA CAA GTA ACT GTT AGG TTC CCT
Phe Ser Glu Val Trp Lys Pro Ser Pro Gln Val Thr Val Arg Phe Pro
      50                  55                  60

GAC AGT GAC TTT AAG GTG TAC AGG TAC AAT GCG GTA TTA GAC CCG CTA
Asp Ser Asp Phe Lys Val Tyr Arg Tyr Asn Ala Val Leu Asp Pro Leu
 65                  70                  75                  80

GTC ACA GCA CTG TTA GGT GCA TTC GAC ACT AGA AAT AGA ATA ATA GAA
Val Thr Ala Leu Leu Gly Ala Phe Asp Thr Arg Asn Arg Ile Ile Glu
                 85                  90                  95

GTT GAA AAT CAG GCG AAC CCC ACG ACT GCC GAA ACG TTA GAT GCT ACT
Val Glu Asn Gln Ala Asn Pro Thr Thr Ala Glu Thr Leu Asp Ala Thr
            100                 105                 110

CGT AGA GTA GAC GAC GCA ACG GTG GCC ATA AGG AGC GCG ATA AAT AAT
Arg Arg Val Asp Asp Ala Thr Val Ala Ile Arg Ser Ala Ile Asn Asn
        115                 120                 125

TTA ATA GTA GAA TTG ATC AGA GGA ACC GGA TCT TAT AAT CGG AGC TCT
Leu Ile Val Glu Leu Ile Arg Gly Thr Gly Ser Tyr Asn Arg Ser Ser
    130                 135                 140

TTC GAG AGC TCT TCT GGT TTG GTT TGG ACC TCT GGT CCT GCA ACC TAG
Phe Glu Ser Ser Ser Gly Leu Val Trp Thr Ser Gly Pro Ala Thr
145                 150                 155                 159
```

SEQ ID NO:17:

```
Met Ser Tyr Ser Ile Thr Thr Pro Ser Gln Phe Val Phe Leu Ser Ser
 1               5                  10                  15

Ala Trp Ala Asp Pro Ile Glu Leu Ile Asn Leu Cys Thr Asn Ala Leu
            20                  25                  30

Gly Asn Gln Phe Gln Thr Gln Gln Ala Arg Thr Val Val Gln Arg Gln
        35                  40                  45

Phe Ser Glu Val Trp Lys Pro Ser Pro Gln Val Thr Val Arg Phe Pro
    50                  55                  60

Asp Ser Asp Phe Lys Val Tyr Arg Tyr Asn Ala Val Leu Asp Pro Leu
65                  70                  75                  80

Val Thr Ala Leu Leu Gly Ala Phe Asp Thr Arg Asn Arg Ile Ile Glu
                85                  90                  95

Val Glu Asn Gln Ala Asn Pro Thr Thr Ala Glu Thr Leu Asp Ala Thr
            100                 105                 110

Arg Arg Val Asp Asp Ala Thr Val Ala Ile Arg Ser Ala Ile Asn Asn
        115                 120                 125

Leu Ile Val Glu Leu Ile Arg Gly Thr Gly Ser Tyr Asn Arg Ser Ser
    130                 135                 140

Phe Glu Ser Ser Ser Gly Leu Val Trp Thr Ser Gly Pro Ala Thr
145                 150                 155             159
```

SEQ ID NO: 19: attatgcatc ttgactacct aggtccaaac caaac
SEQ ID NO: 20: gtcatatgtt ccatctgcag agcagatctt ggaattcgtt aagcaaatct cgagtcagta actata
SEQ ID NO: 21: tatagttact gactcgagat ttgcttaacg aattccaaga tctgctctgc agatggaaca tatgac
SEQ ID NO: 22: cgacctaggt gatgacgtca tagcaattaa cgt
SEQ ID NO: 23: taattgctat gacgtcatca cctaggtcga cgt

EXAMPLE 1

Propagation and purification of the U1 strain of TMV

The TMV VCP fusion vectors described in the following examples are based on the U1 or wild-type TMV strain and are therefore compared to the parental virus as a control. *Nicotiana tabacum cv Xanthi* (hereafter referred to as tobacco) was grown 4-6 weeks after germination, and two 4-8 cm expanded leaves were inoculated with a solution of 50 µg/ml TMV U1 by pipetting 100 µl onto carborundum dusted leaves and lightly abrading the surface with a gloved hand. Six

tobacco plants were grown for 27 days post inoculation accumulating 177 g fresh weight of harvested leaf biomass not including the two lower inoculated leaves. Purified TMV U1 Sample ID No. TMV204.B4 was recovered (745 mg) at a yield of 4.2 mg of virion per g of fresh weight by two cycles of differential centrifugation and precipitation with PEG according to the method of Gooding *et al.* (1967). Tobacco plants infected with TMV U1 accumulated greater than 230 micromoles of CP per kg of leaf tissue.

EXAMPLE 2

Production of a malarial B cell epitope fused to the surface loop region of the TMV VCP

The monoclonal antibody NVS3 was made by immunizing a mouse with irradiated *P. vivax* sporozoites. NVS3 mAb passively transferred to monkeys provided protective immunity to sporozoite infection with this human parasite. Using the technique of epitope-scanning with synthetic peptides, the exact amino acid sequence present on the *P. vivax* sporozoite surface and recognized by NVS3 was defined as AGDR (SEQ ID NO. 3). The epitope AGDR is contained within a repeating unit of the circumsporozoite (CS) protein (Charoenvit *et al.*, 1991a), the major immunodominant protein coating the sporozoite. Construction of a genetically modified tobamovirus designed to carry this malarial B-cell epitope fused to the surface of virus particles is set forth herein.

Construction of plasmid pBGC291

The 2.1 kb EcoRI-PstI fragment from pTMV204 described in Dawson, W., *et al.* (1986) was cloned into pBstSK- (Stratagene Cloning Systems) to form pBGC11. A 0.27 kb fragment of pBGC11 was PCR amplified using the 5' primer TB2ClaI5' (SEQ ID NO: 4) and the 3' primer CP.ME2+ (SEQ ID NO: 5)(see Table 1). The 0.27 kb amplified product was used as the 5' primer and C/0AvrII (SEQ ID NO: 6)(see Table 1) was the 3' primer for PCR amplification. The amplified product was cloned into the SmaI site of pBstKS+ (Stratagene Cloning Systems) to form pBGC243.

To eliminate the BstXI and SacII sites from the polylinker, pBGC234 was formed by digesting pBstKS+ (Stratagene Cloning Systems) with BstXI followed by treatment with T4 DNA Polymerase and self-ligation. The 1.3 kb HindIII-KpnI fragment of pBGC304 was cloned into pBGC234 to form pBGC235. pBGC304 is also named pTMV304 (ATCC deposit 45138).

The 0.3 kb PacI-AccI fragment of pBGC243 was cloned into pBGC235 to form pBGC244. The 0.02 kb polylinker fragment of pBGC243 (SmaI-EcoRV) was removed to form pBGC280. A 0.02 kb synthetic PstI fragment encoding the *P. vivax* AGDR repeat was formed by annealing AGDR3p (SEQ ID NO: 7) with AGDR3m (SEQ ID NO: 8) (see Table 1) and the resulting double

stranded fragment was cloned into pBGC280 to form pBGC282. The 1.0 kb NcoI-KpnI fragment of pBGC282 was cloned into pSNC004 to form pBGC291.

The CP sequence of the virus TMV291 produced by transcription of plasmid pBGC291 *in vitro* is listed in (SEQ ID NO: 9) (the amino acid sequence alone is listed in SEQ ID NO: 10) (see Table 1). The epitope (AGDR)3 is calculated to be approximately 6.2% of the weight of the virion.

Propagation and purification of the epitope expression vector.

Infectious transcripts were synthesized from KpnI-linearized pBGC291 using T7 RNA polymerase and cap (7mGpppG) according to the manufacturer (New England Biolabs). An increased quantity of recombinant virus was obtained by passaging and purifying Sample ID No. TMV291.1B1 as described in Example 1. Twenty tobacco plants were grown for 29 days post inoculation, accumulating 1060 g fresh weight of harvested leaf biomass not including the two lower inoculated leaves. Purified Sample ID TMV291.1B2 was recovered (474 mg) at a yield of 0.4 mg virion per g of fresh weight. Therefore, 25 μg of 12-mer peptide was obtained per g of fresh weight extracted. Tobacco plants infected with TMV291 accumulated greater than 21 micromoles of peptide per kg of leaf tissue.

Product analysis: The conformation of the epitope AGDR contained in the virus TMV291 is specifically recognized by the monoclonal antibody NVS3 in ELISA assays (Figure 4). By Western blot analysis, NVS3 cross-reacted only with the TMV291 cp fusion at 18.6 kD and did not cross-react with the wild type or CP fusion present in TMV261. The genomic sequence of the epitope coding region was confirmed by directly sequencing viral RNA extracted from Sample ID No. TMV291.1B2.

EXAMPLE 3

Production of a malarial B-cell epitope genetically fused to the C terminus of the TMV VCP

Significant progress has been made in designing effective subunit vaccines using rodent models of malarial disease caused by nonhuman pathogens such as *P. yoelii* or *P. berghei*. The monoclonal antibody NYS1 recognizes the repeating epitope QGPGAP (SEQ ID NO: 11), present on the CS protein of *P. yoelii*, and provides a very high level of immunity to sporozoite challenge when passively transferred to mice (Charoenvit, Y., *et al.* 1991b). Construction of a genetically modified tobamovirus designed to carry this malarial B-cell epitope fused to the surface of virus particles is set forth herein.

Construction of plasmid pBGC261

A 0.5 kb fragment of pBGC11, was PCR amplified using the 5' primer TB2ClaI5' (SEQ ID NO: 4) and the 3' primer C/0AvrII (SEQ ID NO: 6). The amplified product was cloned into the SmaI site of pBstKS+ (Stratagene Cloning Systems) to form pBGC218.

pBGC219 was formed by cloning the 0.15 kb AccI-NsiI fragment of pBGC218 into pBGC235. A 0.05 kb synthetic AvrII fragment was formed by annealing PYCS.1p (SEQ ID NO: 12) with PYCS.1m (SEQ ID NO: 13) (see Table 1) and the resulting double stranded fragment, encoding the leaky-stop signal and the *P. yoelii* B-cell malarial epitope, was cloned into the AvrII site of pBGC219 to form pBGC221. The 1.0 kb NcoI-KpnI fragment of pBGC221 was cloned into pBGC152 to form pBGC261.

The virus TMV261, produced by transcription of plasmid pBGC261 *in vitro*, contains a leaky stop signal at the C terminus of the CP gene and is therefore predicted to synthesize wild-type and recombinant coat proteins at a ratio of 20:1. The recombinant TMV VCP fusion synthesized by TMV261 is listed in (SEQ ID NO: 15) (see Table 1) with the stop codon from the wild type mutated to a codon for the amino acid Tyr (amino acid residue 160). The wild-type sequence, synthesized by the same virus, is listed in (SEQ ID NO: 17 (see Table 1). The epitope $(QGPGAP)_2$ (SEQ ID NO: 11) at the C-terminus is calculated to be present at 0.3% of the weight of the virion.

Propagation and purification of the epitope expression vector.

Infectious transcripts were synthesized from KpnI-linearized pBGC261 using SP6 RNA polymerase and cap (7mGpppG) according to the manufacturer (Gibco/BRL Life Technologies).

An increased quantity of recombinant virus was obtained by passaging and purifying Sample ID No. TMV261.B1b as described in Example 1. Six tobacco plants were grown for 27 days post inoculation, accumulating 205 g fresh weight of harvested leaf biomass not including the two lower inoculated leaves. Purified Sample ID No. TMV261.1B2 was recovered (252 mg) at a yield of 1.2 mg virion per g of fresh weight. Therefore, 4 μg of 12-mer peptide was obtained per g of fresh weight extracted. Tobacco plants infected with TMV261 accumulated greater than 3.9 micromoles of peptide per kg of leaf tissue.

Product analysis. The content of the epitope QGPGAP in the virus TMV261 was determined by ELISA with monoclonal antibody NYS1 (Figure 5). From the titration curve, 50 μg/ml of TMV261 gave the same O.D. reading (1.0) as 0.2 ug/ml of $(QGPGAP)_2$. The measured value of approximately 0.4% of the weight of the virion as epitope is in good agreement with the calculated value of 0.3%. By Western blot analysis, NYS1 cross-reacted only with the TMV261 CP fusion at 19 kD and did not cross-react with the wild-type CP or CP fusion present in TMV291.

The genomic sequence of the epitope coding region was confirmed by directly sequencing viral RNA extracted from Sample ID. No. TMV261.1B2.

EXAMPLE 4

Production of a malarial CTL epitope genetically fused to the C terminus of the TMV VCP

Malarial immunity induced in mice by irradiated sporozoites of *P. yoelii* is also dependent on CD8+ T lymphocytes. Clone B is one cytotoxic T lymphocyte (CTL) cell clone shown to recognize an epitope present in both the *P. yoelii* and *P. berghei* CS proteins. Clone B recognizes the following amino acid sequence; SYVPSAEQILEFVKQISSQ (SEQ ID NO: 18) and when adoptively transferred to mice protects against infection from both species of malaria sporozoites (Weiss *et al.*, 1992). Construction of a genetically modified tobamovirus designed to carry this malarial CTL epitope fused to the surface of virus particles is set forth herein.

Construction of plasmid pBGC289.

A 0.5 kb fragment of pBGC11 was PCR amplified using the 5' primer TB2ClaI5' (SEQ ID NO: 4) and the 3' primer C/-5AvrII (SEQ ID NO: 19) (see Table 1). The amplified product was cloned into the SmaI site of pBstKS+ (Stratagene Cloning Systems) to form pBGC214.

pBGC215 was formed by cloning the 0.15 kb AccI-NsiI fragment of pBGC214 into pBGC235. The 0.9 kb NcoI-KpnI fragment from pBGC215 was cloned into pBGC152 to form pBGC216.

A 0.07 kb synthetic fragment was formed by annealing PYCS.2p (SEQ ID NO: 20) with PYCS.2m (SEQ ID NO: 21) (see Table 1) and the resulting double stranded fragment, encoding the *P. yoelii* CTL malarial epitope, was cloned into the AvrII site of pBGC215 made blunt ended by treatment with mung bean nuclease and creating a unique AatII site, to form pBGC262. A 0.03 kb synthetic AatII fragment was formed by annealing TLS.1EXP (SEQ ID NO: 22) with TLS.1EXM (SEQ ID NO: 23) (see Table 1) and the resulting double stranded fragment, encoding the leaky-stop sequence and a stuffer sequence used to facilitate cloning, was cloned into AatII digested pBGC262 to form pBGC263. pBGC262 was digested with AatII and ligated to itself removing the 0.02 kb stuffer fragment to form pBGC264. The 1.0 kb NcoI-KpnI fragment of pBGC264 was cloned into pSNC004 to form pBGC289.

The virus TMV289 produced by transcription of plasmid pBGC289 *in vitro*, contains a leaky stop signal resulting in the removal of four amino acids from the C terminus of the wild-type TMV CP gene and is therefore predicted to synthesize a truncated CP and a CP with a CTL epitope fused at the C terminus at a ratio of 20:1. The recombinant TMV VCP/CTL epitope fusion present in TMV289 (encoded in the nucleotide sequence listed in SEQ ID NO: 24) is listed in SEQ ID NO:

25 (see Table 1) with the stop codon decoded as the amino acid Y (amino acid residue 156). The wild-type sequence minus four amino acids from the C terminus is listed in SEQ ID NO: 26 (the amino acid sequence alone is listed in SEQ ID NO: 27) (see Table 1). The amino acid sequence of the CP of virus TMV216 produced by transcription of the plasmid pBGC216 *in vitro*, is also truncated by four amino acids. The epitope SYVPSAEQILEFVKQISSQ (SEQ ID NO: 18) is calculated to be present at approximately 0.5% of the weight of the virion using the same assumptions confirmed by quantitative ELISA analysis of the readthrough properties of TMV261 in Example 3.

Propagation and purification of the epitope expression vector.

Infectious transcripts were synthesized from KpnI-linearized pBGC289 using T7 RNA polymerase and cap (7mGpppG) according to the manufacturer (New England Biolabs). An increased quantity of recombinant virus was obtained by passaging Sample ID No. TMV289.11B1a as described in Example 1. Fifteen tobacco plants were grown for 33 days post inoculation accumulating 595 g fresh weight of harvested leaf biomass not including the two lower inoculated leaves. Purified Sample ID. No. TMV289.11B2 was recovered (383 mg) at a yield of 0.6 mg virion per g of fresh weight. Therefore, 3 μg of 19-mer peptide was obtained per g of fresh weight extracted. Tobacco plants infected with TMV289 accumulated greater than 1.4 micromoles of peptide per kg of leaf tissue.

Product analysis: Partial confirmation of the sequence of the epitope coding region of TMV289 was obtained by restriction digestion analysis of PCR amplified cDNA using viral RNA isolated from Sample ID. No. TMV289.11B2. The presence of proteins in TMV289 with the predicted mobility of the CP fusion at 20 kD and the truncated CP at 17.1 kDa was confirmed by denaturing PAGE.

EXAMPLE 5.

Construction of pJL 60.3

To facilitate cloning of TMV U1 CP fusions into an infectious TMV U1 cDNA backbone, the vector pJL 60.3 was constructed. The plasmid pJL 60.3 contains a full length infectious clone of TMV U1 with a small multiple cloning site polylinker:

taaatattcttaagccagtagtatgggatatccagtggtatgggatcctacagtatc (SEQ ID NO: 28)

containing two BstXI sites, ccagtagtatgg (SEQ ID NO: 29) and ccagtggtatgg (SEQ ID NO: 30), separated by a unique EcoRV site (GATATC), between the stop codon of the 30K protein gene and the start codon of the U1 CP.

To construct pJL 60.3, a 0.7 kb DNA fragment comprising the TMV U1 CP and 3' UTS was PCR amplified from pBTI 801 using the following primers:

kinased 5' primer JAL 72

tgggatatccagtggtatgggatcctacagtatacactactccatctcag (SEQ ID NO: 31) and

3' primer JON 56

cgcgtacctgggcccctaccgggggtaacg (SEQ ID NO: 32).

pBTI 801 contains a full length infectious clone TMV U1, under the control of the T7 promoter sequence, in a pUC based plasmid. A KpnI restriction enzyme site lies at the 3' end of the viral cDNA, immediately followed by a self-processing ribozyme sequence from satellite tobacco ringspot virus RNA. The presence of this self-processing ribozyme downstream of the TMV 3' end allows for the transcription of the TMV cDNA without prior linearization of the plasmid template DNA (e.g., with KpnI).

A 0.3 kb fragment of pBTI 801 was then PCR amplified using the following primers:

5' primer JON 52 (TMV U1 nts 5456-5482):

ggcccatggaacttacagaagaagtcg (SEQ ID NO: 33)

kinased 3' primer JAL 73

ctggatatcccatactactggcttaagaatatttaaaacgaatccgattcggcgaca (SEQ ID NO: 34).

The 0.7 kb PCR product, containing the EcoRV and BstXI site ccagtggtatgg (SEQ ID NO: 30) upstream of the U1 CP ORF and 3' UTS, was then ligated to the 0.3 bp PCR products (which contained the 3' end of the TMV 30K protein gene and the BstXI site ccagtagtatgg (SEQ ID NO: 29) downstream of the 30K protein stop codon. The product of this ligation reaction was then used in a PCR with 5' primer JON 52 (shown above) and 3' primer JON56 (shown above) to generate a 1 kb PCR product. That product was digested with PacI and NcoI, and the digested DNA was electrophoresed on an agarose gel. The NcoI site is contained within the primer sequence of JON 52, and the PacI site is a unique restriction site in the TMV U1 CP gene sequence. The 0.4 kb PacI-NcoI fragment was then isolated from an agarose gel and ligated into a PacI-NcoI digested 8.8 kb fragment of pBTI 801 to generate pJL 60.3.

Again, the relevant feature of pJL 60.3 for the construction of pBTI 2149 and pBTI 2150 is the existence of the BstXI site ccagtagtatgg (SEQ ID NO: 29) between the TMV 30K stop codon and the CP start codon.

EXAMPLE 6

Construction of plasmid pBTI 2149

A 0.7 kb DNA fragment comprising the TMV U1 coat protein (CP) and 3' UTS was PCR amplified from p BTI 801 using the following primers:

5' primer JAL 149

cctgggccagtagtatgggttcagatggtgctgtacaaccagatggaggtcaaccagctgtatcttacag tatcactactccatctcagtt (SEQ ID NO: 35)

3' primer JON 56 (shown above)

JAL 149 contains the BstXI restriction enzyme site (underscored) for cloning purposes and the coding sequence for the parovirus epitope MGSDGAVQPDGGQPAV (SEQ ID NO: 36) and TMV U1 nts 5715-5743. The amplified product comprising the parvovirus epitope fused to the U1 CP gene was digested with KpnI and BstXI and ligated into the 8.4 kb KpnI-BstXI fragment of pJL 60.3 to generate pBTI 2149. Plasmid vectors pBTI 2149 encodes the recombinant virus having a fusion protein of MGSDGAVQPDGGQPAV (SEQ ID NO: 36) fused to the N-terminus of the coat protein. Plasmid vectors pBTI 2149 was deposited at the ATCC on February 17, 2000, under the Budapest Treaty (ATCC accession no. PTA-1403).

EXAMPLE 7

Construction of plasmid pBTI 2150

A 0.7 kb DNA fragment comprising the TMV U1 CP and 3' UTS was PCR amplified from p801 (basically pTMV 204) using the following primers:

5' primer JAL 150

cctgggccagtagtatgggttcagatggtgctgtacaaccagatggaggtcaaccagctgtatcttacag tatcactactccatctcagtt (SEQ ID NO: 37)

3' primer JON 56. (shown above)

(The "forward" primer JAL 150 contains a BstXI restriction enzyme site (underscored above) for cloning purposes, the coding sequence for the parovirus epitope MGQPDGGQPAVRNERAT (SEQ ID NO: 38) and TMV U1 nts 5718-5743.) The amplified product comprising the parvovirus epitope fused to the U1 CP gene was digested with KpnI and BstXI and ligated into the 8.4 kb KpnI-BstXI fragment of pJL 60.3 to generate pBTI 2150. Plasmid vectors pBTI 2150 encodes the recombinant virus having a fusion protein of MGQPDGGQPAVRNERAT (SEQ ID NO: 38) fused to the N-terminus of the CP. Plasmid vectors pBTI 2150 was deposited at the ATCC on February 17, 2000, under the Budapest Treaty (ATCC accession no. PTA-1404).

EXAMPLE 8

Production of Virus TMV 149

The virus TMV 149 was produced by transcription of plasmid pBTI 2149. Infectious transcripts were synthesized from transcription reactions with T7 RNA polymerase in the presence of cap analog (7mGpppG) (New England Biolabs) according to the manufacturer's instructions. Transcripts were used to inoculate *N. benthamiana* and *N. tabacum* leaves which had been lightly dusted with carborundum (silicon carbide 400 mesh, Aldrich).

EXAMPLE 9

Production of Virus TMV 150

The virus TMV 150 was produced by transcription of plasmid pBTI 2150. Infectious transcripts were synthesized from transcription reactions with T7 RNA polymerase in the presence of cap analog (7mGpppG) (New England Biolabs) according to the manufacturer's instructions. Transcripts were used to inoculate *N. benthamiana* and *N. tabacum* leaves which had been lightly dusted with carborundum (silicon carbide 400 mesh, Aldrich).

EXAMPLE 10

Extraction and Purification of TMV CP Fusion Virions

The two TMV coat fusion constructs, TMV149 and TMV150, were expressed in and extracted from *N. benthamiana* and/or *N. tabacum* using a pH-heat or PEI extraction method as described below, and in Table l. Virus preparations were characterized using MALDI-TOF (Example 11; see Table 3). Based upon the product masses determined by MALDI and PAGE analysis, a proteolytic degradation profile was determined for each construct for any given host plant or extraction method used to produce the coat fusion product (See Tables 3 and 4).

pH-Heat Extractio: *N. benthamiana* or *N. tabacum* cv MD609, produced in growth rooms, were inoculated with TMV derivatives containing parvovirus epitopes fused to the N terminus of the coat protein (TMV149 and TMV150 fusions). Plants were harvested 2.5-5 weeks post inoculation after systemic spread of the virus Leaf and stalk tissue (150 g) was macerated in a 1 L Waring® blender for 2.0 minutes at the high setting with 300 ml of chilled, 0.04% $Na_2S_2O_5$. The macerated material was strained through four layers of cheesecloth to remove fibrous material. The resultant "green juice" was adjusted to a pH of 5.0 with H_3PO_4. The pH adjusted green juice was heated to 47°C and held at this temperature for 5 minutes and then cooled to 15°C. The heat-treated green juice was centrifuged at 6,000 x G for 3 minutes resulting in two fractions, supernatant 1 and pellet 1. The pellet 1 fraction was resuspended in distilled water using a volume of water equivalent to l/z of the initial green juice volume. The resuspended pellet 1 was adjusted

to a pH of 7.5 with NaOH and centrifuged at 6,000 x G for 3 minutes resulting in two fractions, supernatant 2 and pellet 2. Virus was precipitated from both supernatant fractions 1 and 2 by the addition of PEG 6,000 and NaCl (4% by volume). After incubation at 4°C (1 hour), precipitated virus was recovered by centrifugation at 10,000 x G for 10 minutes. The virus pellet was resuspended in 10 mM $NaKPO_4$ buffer, pH 7.2 and clarified by centrifugation at 10,000 x G for 3 minutes. The clarified virus preparation was precipitated a second time by the addition of PEG 6,000 and NaCl (4% by volume). Precipitated virus was recovered by centrifugation as described above. Virus yields are shown in Table 2.

PEI Extraction: *N. benthamiana* or *N. tabacum* cv MD609, produced in growth rooms, were inoculated with TMV derivatives containing parvovirus epitopes fused to the N terminus of the CP (TMV149 and TMV150 fusions). Plants were harvested 2.5-5 weeks post inoculation after systemic spread of the virus. Leaf and stalk tissue (150 g) was macerated in a 1 L Waring blender for 2.0 minutes at the high setting with 300 ml of chilled, 50 mM Tris, pH 7.5, 2 mM EDTA and 0. 1% β-mercaptoethanol. The macerated material was strained through four layers of cheesecloth to remove fibrous material. The resultant "green juice" was adjusted to 0.1% PEI (Sigma, St. Louis, MO) by the addition of a 10% PEI W/V stock solution. The PEI treated green juice was stirred for 30 minutes, (4°C) and then centrifuged at 3,000 x G for 5 minutes resulting in two fractions, supernatant 1 and pellet 1. The pellet 1 fraction was resuspended in distilled water using a volume of water equivalent to ½ of the initial green juice volume. The resuspended pellet 1 was adjusted to a pH of 7.5 with NaOH and centrifuged at 6,000 x G for 3 minutes resulting in two fractions, supernatant 2 and pellet 2. Virus was precipitated from both supernatant fractions 1 and 2 by the addition of PEG 6,000 and NaCl (4% by volume). After incubation at 4°C (1 hour), precipitated virus was recovered by centrifugation at 10,000 x G for 10 minutes. The virus pellet was resuspended in 10 mM $NaKPO_4$ buffer, pH 7.2 and clarified by centrifugation at 10,000 x G for 3 minutes. The clarified virus preparation was precipitated a second time by the addition of PEG 6,000 and NaCl (4% by volume). Precipitated virus was recovered by centrifugation as described above. Virus yields are shown in Table 2.

The yield of epitope specific virus particles is dependent upon the species of plant used as the virus host and method of extraction. TMV149 yielded the highest quantity of virus when produced in *N. benthamiana* and extracted using the pH-heat method. In addition, the TMV149 particles partitioned primarily into supernatant l. Negligible yields of TMV149 were observed when the PEI method was employed. TMV150 yielded the highest quantity of virus when

produced in *N. benthamiana* and extracted using the PEI method. TMV150 partitioned into both supernatant 1 and 2 (60% and 40%, respectively) when extracted by the pH-heat method.

Table 2. Virus Yield

Vector	Host Plant	Extraction Method	Virus Yield*
TMV149	*N. benthamiana*	pH-Heat, Supernatant 1	0.3929
TMV149	*N. benthamiana*	pH-Heat, Supernatant 2	0.0396
TMV149	*N. benthamiana*	PEI, Supernatant 1	0.0005
TMV149	*N. benthamiana*	PEI, Supernatant 2	-
TMV149	*N. tabacum*	pH-Heat, Supernatant 1	0.0488
TMV149	*N. tabacum*	pH-Heat, Supernatant 2	0.0376
TMV149	*N. tabacum*	PEI, Supernatant 1	-
TMV149	*N. tabacum*	PEI, Supernatant 2	-
TMV150	*N. benthamiana*	pH-Heat, Supernatant 1	1.2274
TMV150	*N. benthamiana*	PEI, Supernatant 2	0.8860
TMV150	*N. benthamiana*	PEI, Supernatant 1	1.5369
TMV150	*N. tabacum*	PEI, Supernatant 2	-
TMV150	*N. tabacum*	pH-Heat, Supernatant 1	0.321
TMV150	*N. tabacum*	PEI, Supernatant 1	0.0368
TMV150	*N. tabacum*	PEI, Supernatant 2	0.0001

* Virus yields are expressed as mg virus per g fresh weight, plant tissue extracted and were determined spectrophotometrically (absorbance at A260). All values were derived from the initial PEG precipitates.

EXAMPLE 11

Analysis of CP Fusions by MALDI

PEG precipitated, resuspended virus preparations were diluted in 50% acetonitrile and further diluted 1:1 with sinapinic acid (Aldrich, Milwaukee, WI). The sinapinic acid was prepared at a concentration of 10 mg/ml in 0.1% aqueous triflouroacetic acid/acetonitrile (70/30 by volume). The sinapinic acid treated sample (1.0 μi) was applied to a stainless steel MALDI plate surface and allowed to air dry at room temperature. MALDI-TOF mass spectra were obtained with a PerSeptive Biosystems DE-PRO (Houston, TX) operated in the linear mode. A pulsed laser operating at 337 rim was used in the delayed extraction mode for ionization. An acceleration voltage of 25 kV with a 90% grid voltage and a 0.1% guide wire voltage was used. Approximately 100 scans were acquired and averaged over the mass range 2-156 kDa with a low mass gate of 2 kDa. Ion source and mirror pressures were approximately 1.2×10^{-7} and 1.6×10^{-7} Torr, respectively. All spectra were mass calibrated with a single-point fit using horse apomyoglobin (16,952 Da).

The results presented in Tables 3 and 4 indicate effects of host species, extraction method and extraction timing on the proteolysis of N-terminal TMV CP fusions. In all cases, the terminal

Met residue is removed from all fusions, as is the case with native CP. The N-terminal glycine residue is removed from 40-60% of the TMV149 fusions. Extractions (pH-heat) performed on TMV149 and 150 produced in 17 day post inoculated *N. tabacum*, resulted in the most complex and greatest degree of proteolytic activity. The differences in proteolytic degradation may reflect both qualitative and quantitative differences in proteases present in different plant species or at different plant development periods. The PEI extraction of TMV150 proved to be protective, resulting in negligible degradation relative to the pH-heat extraction *(N. tabacum* host).

Table 3. Product Mass Characterization

Plant Host/Vector	Days Post Inoculation	Extraction Method and Fraction	Product Mass (MALDI) Daltons*,**
N. benthamiana / TMV149	17	pH-Heat Supernatant 1	18,822 (50%); 18,766 (50%) **
N. tabacum / TMV149	17	pH-Heat Supernatant 1	18,823 (40%); 18,762 (40%): 18,564 (<2%); 18,509 (<2%); 18,442 (2%); 18,329 (<2%); 17,993 (10%); 17,935 (2%)
N. tabacum / TMV149	35	pH-Heat, Supernatant 1	18,812 (60%); 18,752 (40%)
N. benthamiana / TMV150	17	pH-Heat, Supernatant 1	19,025 (>95%); 17,964 (<5%)
N. benthamiana / TMV150	17	PEI, Supernatant 1	19,029 (>95%); 17,980 (<5%)
N. tabacum / TMV150	17	pH-Heat, Supernatant 1	19,020 (60%); 17,956 (40%)**
N. tabacum / TMV150	35	pH-Heat, Supernatant 1	19,020 (80%); 17,956 (20%)
N. tabacum / TMV150	17	PEI, Supernatant 1	19,021 (>95%); 17,957 (<5%)

* The number in parentheses is the approximate percentage of coat fusion present at that particular mass (based upon the analysis of fusion proteins separated by PAGE and stained with Coomassie blue).
** Mass is corrected for Sodium ions (23 Da).

Table 4. Proteolytic Degradation Profiles

TMV149		MW (Da)
GSDGAVQPDGGQPAVSYSITTPSQ	(SEQ ID NO: 39)	18,816.5
SDGAVQPDGGQPAVSYSITTPSQ	(SEQ ID NO: 40)	18,759.5
GAVQPDGGQPAVSYSITTPSQ	(SEQ ID NO: 41)	18,557.4
AVQPDGGQPAVSYSITTPSQ	(SEQ ID NO: 42)	18,500.4
VQPDGGQPAVSYSITTPSQ	(SEQ ID NO: 43)	18,429.4
QPDGGQPAVSYSITTPSQ	(SEQ ID NO: 44)	18,330.3
GGQPAVSYSITTPSQ	(SEQ ID NO: 45)	17,990.2
GQPAVSYSITTPSQ	(SEQ ID NO: 46)	17,933.1
TMV150		
GQPDGGQPAVRNERATYSITTPSQ	(SEQ ID NO: 47)	19,027.7
NERATYSITTPSQ	(SEQ ID NO: 48)	17,965.1

Amino acids designated in bold are the natural N-terminal residues resident on TMV VCP.

EXAMPLE 12

Virion Purification and Formulation for Use in Animal Studies

PEG precipitated virion preparations (see Table 5) were resuspended in water for injection (WFI) at a concentration of 1.0 mg virus per 1.0 ml WFI. All laboratory ware used to process the virus preparations was baked at 225°C for 18 hours. The resuspended virus preparation was solvent-extracted with chloroform and 1-butanol (8% by volume) by intermittent shaking for 1 hour at room temperature. Phases were separated by centrifugation at 10,000 x G for 5 minutes. The aqueous phase was frozen in a dry ice/methanol bath and lyophilized overnight until dry. The lyophilized virus preparation was resuspended at a concentration of 5-10 mg virus per 1.0 ml WFI. The resuspended virus preparation was packaged in 10 ml serum vials that were sealed by crimping. Samples selection for further processing was based on both yield and percentage of fusion that remained un-degraded (based on MALDI analysis).

Table 5. TMV Fusions Preparations Processed for Animal Studies

TMV Fusion	Host	Extraction Method
TMV149	*N. benthamiana*	pH-Heat, Supernatant 1
TMV150	*N. benthamiana*	PEI, Supernatant 1

EXAMPLE 13

Vaccine Testing

The parvovirus vaccine, utilizing tobacco plant expressed TMV149 fusion and TMV150 fusion, was tested in young cats for safety and efficacy. The TMV150 fusion expressed on TMV

particles proved to be safe and immunogenic by itself. TMV149 fusion vaccine was somewhat less immunogenic. Cats vaccinated with the TMV150 fusion, the TMV149 fusion or a mixture of the TMV150 fusion and the TMV149 fusion all showed significant protection against a 30% lethal dose of virulent FPV. No adjuvant was required other than what was provided by TMV proteins, some of which are known to act as superantigens (nonspecific immunostimulators). With the development and testing of this particular vaccine, the present inventors have established the usefulness and advantages of the expression system for producing common feline vaccines. The TMV149 fusion and the TMV150 fusion epitopes are the two principal hemagglutinating and neutralizing antibody-inducing antigens on the surface of FPV. The sequences of the two epitopes overlap. Cats immunized with these epitopes will develop virus neutralizing antibodies and will be partially protected against challenge with virulent virus. Therefore, cats were immunized with either TMV149 fusion or TMV150 fusion peptides, or with both, and then monitored for the vaccine's safety, immunogenicity and efficacy. Cats were immunized with 100-200 μg of each peptide, starting at 8-12 weeks of age, and with a second immunization 4 weeks later. They were then challenged orally with a large dose of virulent FPV. Both immunogens appeared completely safe, inducing no fever, depression or local reactions. Antibodies were measured using ELISA. After the second immunization, significant titers of antibodies were detected in ELISA run against whole parvovirus. Cats receiving the TMV149 fusion and the TMV150 fusion gave slightly higher responses than cats immunized with the TMV149 fusion or the TMV150 fusion. After challenge, cats immunized with the TMV150 fusion (either alone or in combination with the TMV149 fusion) appeared to be solidly protected, as evidenced by minimal signs of disease and no mortality, when compared to control cats immunized with TMV alone (that did not express the TMV150 fusion or the TMV149 fusion). It was concluded that the TMV150 fusion peptide, when delivered on TMV particles was a safe and effective vaccine, and moreover, did not require additional adjuvants.

To summarize:

1. Cats immunized with the TMV149 fusion or the TMV150 fusion (100-200μg) made detectable antibody responses as measured by ELISA against whole FPV.
2. The antibody response to 200 μg of the TMV149 fusion or the TMV150 fusion was greater than to 100μg.
3. Cats immunized with a combination of the TMV149 fusion and the TMV150 fusion made better antibody responses than cats immunized with either protein alone.
4. Cats vaccinated with the TMV150 fusion, or the TMV149 fusion and the TMV150 fusion, showed better protection to virulent parvovirus challenge than control cats that were un-

immunized or immunized with TMV. The TMV150 fusion was more protective than the TMV149 fusion.

5. Both the TMV149 fusion and the TMV150 fusion prevented mortality; the TMV150 fusion was more effective at reducing morbidity. The TMV150 fusion-immunized cats were significantly less febrile, showed few clinical signs of illness and were markedly less leukopenic than un-immunized cats or cats immunized with control TMV.

6. Immunity conferred by the TMV150 fusion was not sterilizing, which is typical of killed parvovirus vaccines. Immunized cats showed mild signs of disease but had pronounced immunological memory.

EXAMPLE 14.

Biological Deposit.

The following materials were deposited in accordance with the Budapest Convention on February 5, 2001, at the ATCC (Manassas VA):

TMV U1 strain cDNA: pJL 150/199, ATCC accession # PTA-2983, and

TMV U1 strain cDNA pJL 150/198, ATCC accession # PTA-2984.

Construction of parvo-virus derived peptides fused to the N-terminus of the TMV U1 coat protein via methionine linkage

Table 6 contains amino acid sequences of TMV U1 based CP fusions used or generated. The construction of the TMV150-parvo fusion is described in Example 7. The production of the TMV150 fusion virus is described in Example 9. The parvovirus epitope of interest is underlined. The TMV150 virus was modified to contain the amino acid methionine immediately preceding (TMV150/198 fusion) or following (TMV150/199 fusion) the highly conserved tyrosine (Y) residue of the TMV U1 CP. The presence of the methionine residue renders the peptide susceptible to removal by CNBr cleavage treatment.

Procedures

The modification of the TMV150 fusion virus to generate the TMV150/198 fusion and TMV150/199 fusion was performed using PCR and standard molecular biology procedures. The oligonucleotides JAL198, JAL199, and JAL200 were produced for this experiment. JAL198 [atg tac agt atc act act cca tct cag (SEQ ID NO: 49)] is a forward oligonucleotide that anneals to nine codons from the 5' end of the TMV U1 CP ORF. JAL199 [tac atg agt atc act act cca tct cag (SEQ ID NO: 50)] is a forward nucleotide that mutates the 5' end of the TMV U1 CP ORF to encode YMSITTPSQ (SEQ ID NO. 51). JAL200 [agt agc tct ttc gtt tct tac tgc (SEQ ID NO: 52)] is a reverse

oligonucleotide that anneals to the TMV150 CP fusion at nucleotides 5756-5759, the parvovirus epitope codons for AVRNERAT (SEQ ID NO: 53).

Vector preparation.

The plasmid pBTI801, which contains a full length infectious cDNA of TMV U1 under the control of the T7 RNA polymerase promoter, was digested with the restriction enzymes NcoI and PacI, which cut the TMV U1 cDNA at nucleotides 5459 and 5781, respectively. The digested DNA was phosphatased with calf alkaline phosphatase and electrophoresed through an agarose gel. The approximately 9 kb sized vector fragment was then isolated from the agarose.

Insert Preparation.

Oligonucleotides JAL198 (SEQ ID NO: 40) and JAL199 (SEQ ID NO: 41) were treated with T4 polynucleotide kinase and then used in the following PCR reactions: JAL71 [CGT CGG ccg cac gtg tga tta cgg aca caa tcc g (SEQ ID NO: 54)] and JAL198 using pJL150 template DNA and JAL71 and JAL199 using pBTI 801 template DNA (see Figure 6). JAL71 is a reverse oligonucleotide that anneals to TMV U1 nucleotides 6217-6240. Both PCR reactions amplify up the complete TMV CP ORF, a DNA fragment of approximately 530 bp.

JAL200 and JAL95 [gtc gtc acg ggc gag tgg aac ttg cct (SEQ ID NO: 55)] were used as primers in a PCR reaction of pJL150 template DNA. JAL95 is a forward oligonucleotide that anneals to TMV U1 nucleotides 5119-5145. pJL150 is a TMV-U1 based clone containing the parvovirus epitope codons fused to the 5' end of the U1 CP gene (see Figure 6). Translation of this ORF generates a CP beginning with the amino acid sequence described in Table 6. The PCR product of JAL200 and JAL95 is approximately 660 bp in size.

The JAL71/198 PCR product was then ligated to the JAL200/95 PCR product. Similarly, the JAL71/199 and JAL200/95 PCR products were ligated together. These ligated DNAs were used as templates for PCR reactions using the primers JAL95 and U1 loop. The U1 loop [gtc taa tac cgc att gta c (SEQ ID NO: 56)] is a reverse oligonucleotide that anneals to the TMV U1 CP loop. The resulting PCR product was approximately 800 bp in size.

Both PCR products were extracted with phenol:$CHCl_3$ and precipitated with ammonium acetate and ethanol after PCR, then resuspended in sterile distilled water and finally digested with the restriction enzymes PacI and NcoI. For each digested PCR product, a band of approximately 385 bp, containing the 3' end of the U1 "30K" gene and the mutated parvovirus epitope fused to the 5' end of the U1 CP ORF, was isolated from an agarose gel. This DNA fragment was ligated into the NcoI-PacI digested pBTI801 vector fragment, prepared as described in Example 5. The plasmids which resulted from these two ligations are named pJL150/198 or pJL150/199.

Ligated DNA was transformed into *E. coli* and DNA amplified and prepared from individual transformed DNA colonies. The DNA was transcribed with T7 RNA polymerase in the presence of rNTPs and GpppG cap analog. The transcripts were transfected into protoplasts and the protoplasts cultured in the appropriate liquid medium. Approximately 3 days post-transfection, protoplast extracts were generated and analyzed by SDS-PAGE and Western blotting, using rabbit anti-TMV U1 sera as the primary antibody and goat anti-rabbit (alkaline phosphatase conjugate) as the secondary antibody. The results demonstrated a fusion protein of approximately the expected size was generated by TMV150/198 fusion and TMV150/199 fusion.

Table 6. Amino acid sequence of TMV U1 based CP fusions

Construct name	N-terminal TMV VCP aa sequence
Wild-type U1 CP (SEQ ID NO: 57)	MYSITTPSQ
TMV150 fusion (SEQ ID NO: 58)	MGQPDGGQPAVRNERATYSITTPSQ
TMV150/198 fusion (SEQ ID NO: 59)	MGQPDGGQPAVRNERATMYSITTPSQ
TMV150/199 fusion (SEQ ID NO: 60)	MGQPDGGQPAVRNERATYMSITTPSQ

EXAMPLE 15

Extraction and Purification of TMV VCP Fusion Virions, pH-Heat Extraction

N. benthamiana, produced in a growth room, were inoculated with the TMV derivative containing a Parvo epitope fused to the N-terminus of the CP via a methionine residue (TMV150/198 and YMV150/199 fusions). Initial screening of plants inoculated with the TMV150/198 and TMV150/199 fusions indicated that the TMV150/198 virus produced a higher yield than the TMV150/199 virus. Further analysis of the TMV150/199 fusion was not pursued. Plants inoculated with the TMV150/198 fusion were harvested 2-3 weeks post inoculation after systemic spread of the virus. Leaf and stalk tissue (221 g) was macerated in a 1 L Waring® blender for 2.0 minutes at the high setting with 300 ml of chilled 0.04% $Na_2S_2O_5$. The macerated material was strained through four layers of cheese cloth to remove fibrous material to produce a "green juice". The resultant "green juice" was adjusted to a pH of 5.0 with H_3PO_4. The pH adjusted green juice was heated to 47°C and held at this temperature for 5 minutes and then cooled to 15°C. The heat-treated green juice was centrifuged at 5,000 x G for 5 minutes resulting in two fractions, Supernatant 1 and Pellet 1. The Pellet 1 fraction was resuspended in distilled water using a volume of water equivalent to ½ of the initial "green juice" volume. The resuspended Pellet 1 was adjusted

to a pH of 7.5 with NaOH and centrifuged at 5,000 x G for 5 minutes resulting in two fractions, Supernatant 2 and Pellet 2. Virus was precipitated from both Supernatant 1 and 2 fractions by the addition of PEG 6,000 and NaCl (4% by volume). After incubation at 4°C for 1 hour, precipitated virus was recovered by centrifugation at 10,000 x G for 15 minutes. The virus pellet was resuspended in 10 mM $NaKPO_4$ buffer, pH 7.2 and clarified by centrifugation at 10,000 x G for 10 minutes. The clarified virus preparation was precipitated a second time by the addition of PEG 6,000 and NaCl (4% by volume). Precipitated virus was recovered by centrifugation as described above. PEG purified virion preparations derived from Supernatants 1 and 2 were analyzed by MALDI-TOF as described in Example 11 and mass weights determined (Table 7). Three protein masses were detected corresponding to the predicted full length TMV VCP fusion, a proteolytic degradation product containing an N-terminal arginine residue and a protein containing an N-terminal methionine residue resulting from initiation of translation on an internal methionine or proteolytic degradation (see Table 8 and Figures 7 and 8).

Table 7. Product Mass Characterization

Sample	Product Mass (MALDI) (Daltons)
Example 15, Supernatant 1, PEG2	19,164; 18,105; 17,537
Example 15, Supernatant 2, PEG2	19,188; 18,122; 17551
Example 16, CNBr Pellet 1	19,330; 17,570
Example 16, CNBr, 10 Kd Permeate, Resuspended Lyophilisate	1736, 1720, 1758, 1828
Example 19, Pellet 1, HPLC, 32.5 minutes	19,211; 17,439
Example 19, Supernantant 1, HPLC, 17.4 minutes	1737

Table 8. Coat Fusion Products.

TMV150/198 (TMV VCP residues are bolded)		**MW (daltons)**
GQPDGGQPAVRNERATM**YSITTPSQ**	(SEQ ID NO: 47)	19,159.7
NERATM**YSITTPSQ**	(SEQ ID NO: 48)	18,097.2
M**YSITTPSQ**	(SEQ ID NO: 57)	17,525.9

EXAMPLE 16.

Cyanogen Bromide Cleavage of TMV VCP Fusions

Thirty mg (4.4 mg virus/ml) of the purified TMV150/198 fusion (Supernatant 1-PEG 2 prepared as described in Example 15) was mixed with 21 ml of formic acid and 2.2 ml of di H_2O resulting in 1 mg/ml protein in 70% formic acid. Thirty mg of solid CNBr was added to the reaction mix, dissolved by shaking, and incubated in the dark at room temperature for 6 hours. After the 6 hour incubation, 350 ml of di H_2O was added to the reaction mix, incubated in a dry ice-ethanol bath until frozen and lyophilized to dryness. The lyophilized powder was resuspended in 10 ml di H_2O and centrifuged at 6,000 x G for 5 minutes resulting in a pellet 1 and supernatant 1 fraction. The pellet 1 fraction was washed by resuspension in 10 ml di H_2O and separated by centrifugation at 6,000 x G for 5 minutes resulting in pellet 2 and supernatant 2 fractions. The supernatant 1 and 2 fractions were combined and filtered through a 10 Kd molecular weight cut-off Amicon centricon®. The 10 Kd permeate was incubated in a dry ice-ethanol bath until frozen and lyophilized to dryness. Lyophilized material was resuspended in di H_2O for analysis. CNBr cleaved products were analyzed by MALDI-TOF as described in Example 17 and mass weights determined (Table7). The CNBr pellet 1 contained two products with mass weights of 19,330 and 17,570 Da corresponding to uncleaved and cleaved TMV VCP, respectively (see Figure 9). Both TMV VCP species have an apparent increase in mass that is likely due to acid ester formation. The resuspended 10 Kd permeate lyophilisate contains predominantly a 1736 Da species and minor quantities of 1720, 1758 and 1828 Da species (Figure 10). The 1736 fragment corresponds to the predicted mass of the released parvo peptide sequence containing a carboxy-terminal homoserine. No uncleaved or cleaved TMV VCP was detected in the 10 Kd permeate lyophilisate sample (Fig. 11).

EXAMPLE 17

Analysis of CP Fusions and CNBr Cleaved Fusions by MALDI

MALDI-TOF (sinapinic acid) analysis of products with masses above 5 kDa. Varying concentrations of each sample were diluted 1:1 with sinapinic acid (Aldrich, Milwaukee, WI) matrix, 1 µL was applied to a stainless steel MALDI plate surface and allowed to air dry for analysis. The sinapinic acid was prepared at a concentration of 10 mg/ml in 0.1% aqueous TFA/acetonitrile (70/30 by volume). MALDI-TOF mass spectra were obtained with a PerSeptive Biosystems Voyager DE-PRO (Houston, TX) operated in the linear mode. A pulsed nitrogen laser operating at 337 nm was used in the delayed extraction mode for ionization. An acceleration voltage of 25 kV with a 90% grid voltage and a 0.1% guide wire voltage was used. Approximately

100 scans were acquired and averaged over the mass range of 2-156 kDa. with a low mass gate of 2000. Ion source and mirror pressures were approximately 5×10^{-8} and 3×10^{-8} Torr, respectively. All spectra were mass calibrated with a single-point fit using horse apomyoglobin (16,952 Da).

MALDI-TOF (α-cyano-4-hydroxycinnamic acid) analysis of products with masses below 5 kDa.

Varying concentrations of each sample were diluted 1:1 with recrystallized α-cyano-4-hydroxycinnamic acid (Aldrich, Milwaukee, WI) matrix, 1 μL was applied to a stainless steel MALDI plate surface and allowed to air dry for analysis. The CHCA was prepared at a concentration of 10 mg/ml in 0.1% aqueous TFA/acetonitrile/ethanol (1:1:1 by volume). MALDI-TOF mass spectra were obtained with a PerSeptive Biosystems Voyager DE-PRO (Houston, TX) operated in the reflectron mode. A pulsed nitrogen laser operating at 337 nm was used in the delayed extraction mode for ionization. An acceleration voltage of 20 kV with a 74% grid voltage and a 0.05% guide wire voltage was used. Approximately 100 scans were acquired and averaged over the mass range of 385-8500 Da. with a low mass gate of 350. Ion source and mirror pressures were approximately 5.9×10^{-8} and 2.8×10^{-8} Torr, respectively. All spectra were mass calibrated with a single point fit using Angiotensin I (1,297.51 Da).

Example 18.

N-Terminal Amino Acid Sequence Analysis of CNBr Cleaved and Purified Peptide

N-terminal amino acid sequencing of the resuspended 10 Kd permeate lyophilisate containing the CNBr-released peptide was performed by the University of Michigan Medical School Protein and Carbohydrate Structure Facility. The lyophilized 10 Kd permeate was resuspended in di H_2O at a protein concentration of 2.6 mg protein/ml and sequenced using an automated ABI Model 477 sequenator. The procedure employed standard Edman degradation to sequentially cleave and identify amino acids starting at the amino terminus (N-terminus) of the peptide. The instrument is capable of detecting all 20 common amino acids, as well as several modified forms. It was operated in a liquid-pulse mode. Sequencing was carried out 15 cycles to identify the first 15 amino acids of the peptide. The first 13 amino acids, based upon sequencing, matched the predicted amino acid sequence. After 13 cycles, the repetitive yield dropped below an about that allowed calling residues 14 and 15 with confidence. However, because the first 13 amino acids match the predicted sequence of the peptide, the identity is confirmed.

Example 19.

Separation and Purification of CNBr Cleaved Peptide by HPLC.

Pellet 1 and Supernatant 1 fractions of CNBr cleaved TMV150/198 fusion, as described in Example 16, were separated by HPLC and peak fractions analyzed by MALDI-TOF as described in Example 17. HPLC Separation was performed on a Hewlett-Packard (Agilent Technologies) Model 1100 HPLC with photo diode array detection capabilities. The conditions were as follows:

Column:	0.2 x 250 Vydac Narrowbore 219TP52 Diphenyl Reverse Phase Column, 5 μm particle size
Flow Rate:	0.25 mL/min.
Solvent A:	5% Acetonitrile, 0.1% TFA (0.22 μm-filtered before use)
Solvent B:	95% Acetonitrile, 0.1% TFA, (0.22 μm-filtered before use)
Gradient:	Isocratic 5 minutes in Solvent A, then to 100% Solvent B over 40 minutes; held 5 minutes at 100% Solvent B; 5 minutes then return to initial conditions.
Detection:	UV absorbance at 210nm and 280 nm simultaneously
Injection Volume:	100 μL (containing about 3 μg total protein by BCA assay)
Temperature:	Not controlled (ambient conditions)

The HPLC chromatograms for the separations of the Pellet 1 and Supernatant 1 fractions are shown in Figures 12 and 13, respectively. The major peaks detected for each sample (17.4 minutes-Supernatant 1 and 32.5 minutes-Pellet 1) were collected and analyzed by MALDI (see Table 7). HPLC effectively separated cleaved peptide, eluting at 17.4 minutes, from uncleaved and cleaved TMV VCP that eluted at 32.5 minutes.

Although the invention has been described with reference to the presently preferred embodiments, it should be understood that various modifications can be made without departing from the spirit of the invention.

All publications, patents, patent applications, and web sites citedd herein are incorporated by reference in their entirety as if each individual publication, patent, patent application, or web site was specifically and individually incorporated by reference.

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WHAT IS CLAIMED IS:

1. A polynucleotide encoding a fusion protein capable of being expressed in a plant or a plant cell, comprising:

(a) a polypeptide or peptide of interest, P1, linked to

(b) the N-terminus of a plant viral coat protein, VCP, via

(c) a cleavable linker L,

such that said fusion protein has the formula, reading from the N to the C terminus:

P1--L--VCP.

2. A polynucleotide encoding a fusion protein capable of being expressed in a plant or a plant cell, comprising at least two polypeptides or peptides of interest, P1 and P2, to be produced in a plant cell or plant, wherein

(a) P1 is linked to the N-terminus of a plant VCP via a cleavable linker L,

(b) P2 is linked to the VCP at

(i) the C-terminus of the VCP or

(ii) internally within the sequence of the VCP, or

(ii) at the N-terminus of the VCP but C-terminal to P1,

P2 being linked to the VCP and, optionally, to said P1 by a cleavable linker L such that said fusion protein has a formula, reading from the N to the C terminus, selected from the group consisting of:

P1--L--VCP--L--P2

P1--L--P2--L--VCP and

P1--L--$VCP_{(a)}$-L-P2-L-$VCP_{(b)}$

wherein $VCP_{(a)}$ and $VCP_{(b)}$ are to two fragments of the VCP that flank the internally located P2, and wherein when more than one L is present, each L may be the same or a different linker.

3. A polynucleotide encoding a fusion protein capable of being expressed in a plant or a plant cell, comprising at least two identical or different polypeptide or peptide units of interest, P_m, wherein

(a) a first P_m, P_{m1} is linked to the N-terminus of a plant VCP via a cleavable linker L,

(b) a second P_m, P_{m2} is linked to the N terminus of P_{m1}, optionally via a cleavable linker L,

(c) optionally, one or more additional P_m units, designated P_{m3}, P_{m4}, ... P_{mn} are linked sequentially to the to the preceding P_m unit at its N-terminus, optionally via a cleavable linker, wherein

(i) each of said P_{m1}, P_{m2} ... P_{mn} are the same or different peptides;

(ii) n is between 3 and 100;

(iii) when more than one L is present, each L may be the same or a different linker,

such that said fusion protein has a formula, reading from the N to the C terminus, selected from the group consisting of:

$$P_{m2}\text{-}L_o\text{-}P_{m1}\text{-}L\text{-}VCP$$

$$P_{m3}\text{-}L_o\text{-}P_{m2}\text{-}L_o\text{-}P_{m1}\text{-}L\text{-}VCP$$

$$P_{m4}\text{-}L_o\text{-}P_{m3}\text{-}L_o\text{-}P_{m2}\text{-}L_o\text{-}P_{m1}\text{-}L\text{-}VCP$$

and

$$P_{mn}\text{-}L_o\text{-}\ldots\text{-}P_{m4}\text{-}L_o\text{-}P_{m3}\text{-}L_o\text{-}P_{m2}\text{-}L_o\text{-}P_{m1}\text{-}L\text{-}VCP.$$

wherein L_o indicates that said linker is optional

4 The polynucleotide of any of claims 1-3 wherein L is a sequence of one or more amino acids selected from the group consisting of methionine, glutamate, aspartate, lysine, arginine, phenylalanine, tryptophan, tyrosine, leucine, and the tetrapeptide isoleucine-glutamate-glycine-arginine.

5. The polynucleotide of claim 4 wherein L is methionine.

6. The polynucleotide of any of claims 1-5 wherein the cleavable linker is cleaved by a non-enzymatic chemical agent.

7. The polynucleotide of claim 6 wherein the chemical agent is cyanogen bromide.

8. The polynucleotide of any of claims 1-5 wherein the cleavable linker is cleaved by an enzyme.

9. The polynucleotide of Claim 8 wherein the enzyme is selected from the group consisting of trypsin, chymotrypsin, pepsin, *Staphylococcus aureus* V8 protease, and Factor Xa protease.

10. The polynucleotide of any of claims 1-9 which comprises elements rendering it expressible in a plant cell or a plant.

11 The polynucleotide of any of claims 1-10 wherein the VCP is a coat protein of a single-stranded plus-sense RNA virus.

12. The polynucleotide of Claim 11 wherein the virus is tobacco mosaic virus.

13. The polynucleotide of any of claims 1-12 wherein P1 comprises one or more epitopes useful in a vaccine composition.

14. The polynucleotide of claim 13 wherein said epitope comprises an amino acid sequence selected from the group consisting of

a. MGSDGAVQPDGGQPAV or a fragment thereof; and

b. MGQPDGGQPAVRNERAT or a fragment thereof.

15. The polynucleotide of any of claims 1-13 wherein P1 is longer than 15 amino acids.

16 A recombinant viral nucleic acid comprising the polynucleotide of any of claims 1-15.

17. A recombinant virus particle comprising the recombinant viral nucleic acid of Claim 166.

18. A recombinant plant virus comprising a viral coat protein encoded by the polynucleotide of any of Claims 1-15.

19. A plant cell comprising the polynucleotide of any of claims 1-15 and

20. A plant cell comprising the recombinant viral nucleic acid of Claim 16.

21. A plant cell comprising the recombinant virus particle of Claim 17.

22. A plant cell comprising the recombinant plant virus of Claim 18.

23. The plant cell of any of claims 19-23 expressing said fusion polypeptide.

24. The plant cell of any of claims 19-23 expressing said P1 polypeptide.

25. A plant comprising the plant cell of any of claims 19-24.

26. A method for producing a polypeptide of interest, P1, comprising the steps of:

(a) contacting a plant or a plant cell with a recombinant plant virus nucleic acid that comprises a polynucleotide encoding a fusion polypeptide, which includes a polypeptide of interest P1 linked to the N-terminus of a plant viral coat protein VCP via a cleavable linker L,

(b) growing the plant or the plant cell under conditions that the fusion polypeptide is expressed, and

(c) cleaving the fusion protein

(i) in the plant or plant cell or

(ii) outside the plant or cell after the fusion protein has been at least partially purified or isolated from said plant or cell,

with a cleaving agent that breaks a covalent bond that bonds P1 and the VCP, so that P1 is separated from the VCP;

thereby producing said P1.

27. A method for producing one or two polypeptides of interest, P1 and P2, comprising the steps of:

(a) contacting a plant or a plant cell with a recombinant plant virus nucleic acid that comprises a polynucleotide encoding a fusion polypeptide, which includes P1 and P2 wherein

(i) P1 is linked to the N-terminus of a plant VCP via a cleavable linker L,

(ii) P2 is linked to the VCP at

(1) the C-terminus of the VCP or

(2) internally within the sequence of the VCP, or

(3) at the N-terminus of the VCP but C-terminal to P1,

P2 being linked to the VCP and, optionally, to said P1 by a cleavable linker L

(b) growing the plant or the plant cell under conditions that the fusion polypeptide is expressed, and

(c) cleaving the fusion protein

(i) in the plant or plant cell or

(ii) outside the plant or cell after the fusion protein has been at least partially purified or isolated from said plant or cell,

with a cleaving agent that breaks a covalent bond that bonds P1 and the VCP, P1 and P2 or P2 and the VCP so that P1 and/or P2 is separated from the VCP;

thereby producing said P1, P2 or both.

28. The method of claim 26 or 27 further comprising the step of:

(d) isolating or purifying said P1, P2 or both from said VCP

29. The method of claim 26, 27 or 28 wherein said fusion polypeptide is purified from said plant or cell prior to said cleaving step.

30. A polynucleotide encoding a fusion protein capable of being expressed in a plant or a plant cell, comprising:

(a) a coding region that encodes said fusion protein and includes:

i. a plant viral coat protein (VCP) from a single-stranded plus-sense RNA virus, and fused thereto,

ii. a peptide comprising amino acids MGSDGAVQPDGGQPAV (SEQ ID NO:1) or a fragment thereof; and

(b) a promoter functional in plants that is 5' to the coding region.

31. The polynucleotide of claim 30, wherein the peptide is fused to the N-terminus of the plant viral coat protein.

32. The polynucleotide of claim 30, wherein the peptide is fused to the C-terminus of the plant viral coat protein.

33. The polynucleotide of claim 30, wherein said fusion protein is an internal fusion protein with respect to the coat protein.

34. The polynucleotide of any of claims 30-33, further comprising

(c) a fusion joint having a leaky stop codon from a single-stranded plus-sense RNA virus.

35. The polynucleotide of claim 30, wherein the peptide MGSDGAVQPDGGQPAV or fragment is an antigenic epitope.

36. The polynucleotide of any of claims 30-35, wherein the coat protein is a tobacco mosaic virus coat protein.

37. The polynucleotide of any of claims 30-35 wherein the coat protein is a tobamovirus coat protein.

38. A recombinant plant viral genome comprising the polynucleotide of any of claims 30-37.

39. A recombinant plant virus particle, comprising a genome according to claim 38.

40. A recombinant plant virus having a coat protein encoded by The polynucleotide of any of claims 30-37.

41. A plant cell comprising the polynucleotide of any of claims 30-37.

42. A plant cell comprising a recombinant plant viral genome according to claim 38.

43. A plant cell comprising a recombinant plant virus particle according to claim 10.

44. A plant cell comprising a recombinant plant virus according to claim 40.

45. A plant comprising the polynucleotide of any of claims 30-37.

46. A plant comprising a recombinant plant viral genome according to claim 38.

47. A plant comprising a recombinant plant virus particle according to claim 39.

48. A plant comprising a recombinant plant virus according to claim 40.

49. A polynucleotide encoding a fusion protein capable of being expressed in a plant or a plant cell, comprising:

(a) a coding region that encodes said fusion protein and includes:

i. a plant VCP from a single-stranded plus-sense RNA virus, and fused thereto,

ii. a peptide comprising amino acids MGQPDGGQPAVRNERAT (SEQ ID NO:2) or a fragment thereof; and

(b) a promoter functional in plants that is 5' to the coding region.

50. The polynucleotide of claim 49, wherein the peptide is fused to the N-terminus of the plant viral coat protein.

51. The polynucleotide of claim 49, wherein the peptide is fused to the C-terminus of the plant viral coat protein.

52 The polynucleotide of claim 49, wherein said fusion protein is an internal fusion protein with respect to the coat protein.

53. The polynucleotide of any of claims 49-52, further comprising

(c) a fusion joint having a leaky stop codon from a single-stranded plus-sense RNA virus.

54. The polynucleotide of any of claims 49-53, wherein the peptide MGQPDGGQPAVRNERAT or a fragment is an antigenic epitope.

55. The polynucleotide of any of claims 49-54, wherein the coat protein is a tobacco mosaic virus coat protein.

56. The polynucleotide of claims 49-54 wherein the coat protein is a tobamovirus coat protein.

57. A recombinant plant viral genome comprising the polynucleotide of any of claims 49-56.

58. A recombinant plant virus particle, comprising a genome according to claim 57.

59. A recombinant plant virus, wherein the coat protein is encoded by the polynucleotide of any of claims 49-56.

60. A plant cell comprising the polynucleotide of any of claims 49-56.

61. A plant cell comprising a recombinant plant viral genome according to claim 57.

62. A plant cell comprising a recombinant plant virus particle according to claim 58.

63. A plant cell comprising a recombinant plant virus according to claim 59.

64. A plant comprising the polynucleotide of any of claims 49-56.

65. A plant comprising a recombinant plant viral genome according to claim 57.

66. A plant comprising a recombinant plant virus particle according to claim 58.

67. A plant comprising a recombinant plant virus according to claim 59.

68. An immunochemical reagent comprising a fusion protein capable of being produced in a plant or a plant cell, wherein the fusion protein comprises

(i) a plant VCP from a single-stranded plus-sense RNA virus; and

(ii) a peptide comprising amino acids MGQPDGGQPAVRNERAT or a fragment thereof fused to the N-terminus of the coat protein.

69. A vaccine for the protection of mammals against parvovirus comprising the immunochemical reagent of claim 68.

70. A vaccine according to claim 69 together with a pharmaceutically or veterinarially acceptable carrier or excipient.

71. An immunochemical reagent comprising a recombinant plant virus, wherein at least one capsid of the coat protein is a fusion protein capable of being produced in a plant or a plant cell, wherein the fusion protein comprises

(i) a plant VCP from a single-stranded plus-sense RNA virus; and

(ii) a peptide comprising amino acids MGSDGAVQPDGGQPAV or a fragment thereof fused to the N-terminus of the coat protein.

72. A vaccine for the protection of mammals against parvovirus comprising an immunochemical reagent according to claim 71.

73. A vaccine according to claim 72 together with a pharmaceutically or veterinarially acceptable carrier.

74. A vaccine according to claim 69, wherein the fusion protein is in a recombinant plant virus.

75. A vaccine according to claim 74 wherein said virus is a live virus.

76. A vaccine according to claim 72, wherein the recombinant plant virus is a live virus.

77. A vaccine composition comprising a live recombinant plant virus according to claim 75 and a pharmaceutically or veterinarially acceptable carrier or excipient.

78. A vaccine composition comprising a live recombinant plant virus according to claim 76 and a pharmaceutically or veterinarially acceptable carrier or excipient.

79. A method of making the polynucleotide of any of claims30-37, comprising ligating an oligonucleotide encoding a peptide having the sequence MGSDGAVQPDGGQPAV or a fragment thereof to a VCP gene.

80. A method of making a recombinant plant viral genome comprising

(a) inserting an oligonucleotide encoding a peptide having the sequence MGSDGAVQPDGGQPAV or a fragment thereof into the genome of a single-stranded plus-sense RNA virus so that said oligonucleotide is fused in frame with a plant VCP gene and under the control of a promoter functional in plants; or

(b) ligating the polynucleotide of any of claims 30-37 to the genome of a single-stranded plus-sense RNA virus,

thereby making said recombinant plant viral genome.

81. A method of making the recombinant plant virus that encodes a fusion protein that includes a plant VCP from a single-stranded plus-sense RNA virus and a peptide comprising amino acids MGSDGAVQPDGGQPAV or a fragment thereof, comprising the steps of:

(a) ligating DNA encoding the fusion protein which protein is capable of being expressed in a plant or a plant cell, to a DNA copy of the genome of a single-stranded plussense RNA virus;

(b) transcribing said ligated DNA to RNA; and

(c) infecting a host plant or plant cell with said transcribed RNA,

so that said plant or plant cell makes said recombinant plant virus.

82. A method of making the recombinant plant virus that encodes a fusion protein that includes a plant VCP from a single-stranded plus-sense RNA virus and a peptide comprising amino acids MGSDGAVQPDGGQPAV or a fragment thereof, comprising the steps of;

(a) ligating DNA encoding the fusion protein which protein is capable of being expressed in a plant or a plant cell, to a DNA copy of the genome of a

single-stranded plussense RNA virus, under the control of a promoter that is functional in plants; and

(b) transforming or transfecting a host plant or plant cell with said ligated DNA, so that said DNA is expressed in said plant or plant cell,

whereby said plant or plant cell makes said recombinant plant virus.

83. A method of making a plant cell that produces a fusion protein that includes a plant VCP from a single-stranded plus-sense RNA virus and a peptide comprising amino acids MGSDGAVQPDGGQPAV or a fragment thereof, comprising transforming or transfecting a host plant cell with the plant viral genome of claim 80.

84. A method of making a plant cell that produces a fusion protein that includes a plant VCP from a single-stranded plus-sense RNA virus and a peptide comprising amino acids MGSDGAVQPDGGQPAV or a fragment thereof, comprising infecting a host plant cell with the plant virus of claim 81.

85. A method of making a plant cell that produces a fusion protein that includes a plant VCP from a single-stranded plus-sense RNA virus and a peptide comprising amino acids MGSDGAVQPDGGQPAV or a fragment thereof, comprising infecting a host plant cell with the plant virus of claim 82.

86. A method of making a plant that produces a fusion protein that includes a plant VCP from a single-stranded plus-sense RNA virus and a peptide comprising amino acids MGSDGAVQPDGGQPAV or a fragment thereof, comprising transforming or transfecting a host plant with the plant viral genome of claim 80.

87. A method of making a plant that produces a fusion protein that includes a plant VCP from a single-stranded plus-sense RNA virus and a peptide comprising amino acids MGSDGAVQPDGGQPAV or a fragment thereof, comprising infecting a host plant with the plant virus of claim 81.

88. A method of making a plant that produces a fusion protein that includes a plant VCP from a single-stranded plus-sense RNA virus and a peptide comprising amino acids MGSDGAVQPDGGQPAV or a fragment thereof, comprising infecting a host plant with the plant virus of claim 82.

89. A method of making the polynucleotide of claim 49-56, comprising ligating an oligonucleotide encoding a peptide having the sequence MGQPDGGQPAVRNERAT or a fragment thereof to a VCP gene.

90. A method of making a recombinant plant viral genome comprising

(a) inserting an oligonucleotide encoding a peptide having the sequence MGQPDGGQPAVRNERAT or a fragment thereof into the genome of a single-stranded plus-sense RNA virus so that said oligonucleotide is fused in frame with a plant VCP gene and under the control of a promoter functional in plants; or

(b) ligating the polynucleotide of claim 1 to the genome of a single-stranded plus-sense RNA virus

thereby making said recombinant plant viral genome.

91. A method of making the recombinant plant virus that encodes a fusion protein that includes a plant VCP from a single-stranded plus-sense RNA virus and a peptide comprising amino acids MGQPDGGQPAVRNERAT or a fragment thereof, comprising the steps of;

(a) ligating DNA encoding the fusion protein which protein is capable of being expressed in a plant or a plant cell, to a DNA copy of the genome of a single-stranded plussense RNA virus;

(b) transcribing said ligated DNA to RNA; and

(c) infecting a host plant or plant cell with said transcribed RNA,

so that said plant or plant cell makes said recombinant plant virus.

92. A method of making the recombinant plant virus that encodes a fusion protein that includes a plant VCP from a single-stranded plus-sense RNA virus and a peptide comprising amino acids MGQPDGGQPAVRNERAT or a fragment thereof, comprising the steps of;

(a) ligating DNA encoding the fusion protein which protein is capable of being expressed in a plant or a plant cell, to a DNA copy of the genome of a single-stranded plussense RNA virus, under the control of a promoter that is functional in plants; and

(b) transforming or transfecting a host plant or plant cell with said ligated DNA, so that said DNA is expressed in said plant or plant cell,

whereby said plant or plant cell makes said recombinant plant virus.

94. A method of making a plant cell that produces a fusion protein that includes a plant VCP from a single-stranded plus-sense RNA virus and a peptide comprising amino acids MGQPDGGQPAVRNERAT or a fragment thereof, comprising transforming or transfecting a host plant cell with the plant viral genome of claim 90.

95. A method of making a plant cell that produces a fusion protein that includes a plant VCP from a single-stranded plus-sense RNA virus and a peptide comprising amino acids MGQPDGGQPAVRNERAT or a fragment thereof, comprising infecting a host plant cell with the plant virus of claim 91.

96. A method of making a plant cell that produces a fusion protein that includes a plant VCP from a single-stranded plus-sense RNA virus and a peptide comprising amino acids MGQPDGGQPAVRNERAT or a fragment thereof, comprising infecting a host plant cell with the plant virus of claim 92.

97. A method of making a plant that produces a fusion protein that includes a plant VCP from a single-stranded plus-sense RNA virus and a peptide comprising amino acids MGQPDGGQPAVRNERAT or a fragment thereof, comprising transforming or transfecting a host plant with the plant viral genome of claim 90.

98. A method of making a plant that produces a fusion protein that includes a plant VCP from a single-stranded plus-sense RNA virus and a peptide comprising amino acids MGQPDGGQPAVRNERAT or a fragment thereof, comprising infecting a host plant with the plant virus of claim 91.

99. A method of making a plant that produces a fusion protein that includes a plant VCP from a single-stranded plus-sense RNA virus and a peptide comprising amino acids MGQPDGGQPAVRNERAT or a fragment thereof, comprising infecting a host plant with the plant virus of claim 92.

100. A method of isolating a virus, comprising:

(a) Homogenizing virus-containing plant tissue in $Na_2S_2O_5$;

(b) Straining the homogenate to obtain green juice;

(c) Adjusting the pH of the green juice to 5.0 with acid;

(d) Heating the green juice to about 47°C for a period of about 5 minutes followed by cooling to about 5°C;

(e) Centrifuging the green juice at about 6000 x g for about 3 minutes to obtain a supernatant and pellet;

(f) Precipitating the supernatant in polyethylene glycol and NaCl to obtain a precipitate;

(g) Resuspending the precipitate in water at a concentration of about 1 mg per ml;

(h) Extracting the precipitate in chloroform and butanol and centrifuging the extract

(i) Recovering and lyophilizing the aqueous phase of the centrifuged material;

(j) Resuspending the lyophilized material at a concentration of about 5 to about 10 mg per ml water.

101. A method of isolating a virus, comprising:

(a) Grinding virus-containing plant material in a buffer to obtain a homogenate;

(b) Straining the homogenate to obtain green juice, and adding thereto polyethyleneimine to a concentration of about 0.1% (v/v)

(c) Stirring at about 4°C for about 30 minutes followed by centrifuging ; at about 3000 x g for about 5 minutes to obtain a supernatant;

(d) Precipitating the supernatant in polyethylene glycol and NaCl to obtain a precipitate;

(e) Resuspending the precipitate in water at a concentration of about 1 mg per ml;

(f) Extracting the resuspended material in chloroform and butanol and centrifuging the extracted material;

(g) Recovering and lyophilizing the aqueous phase; and

(h) Resuspending the lyophilized material at a concentration of about 5 to about 10 mg per ml water.

FIG. 1

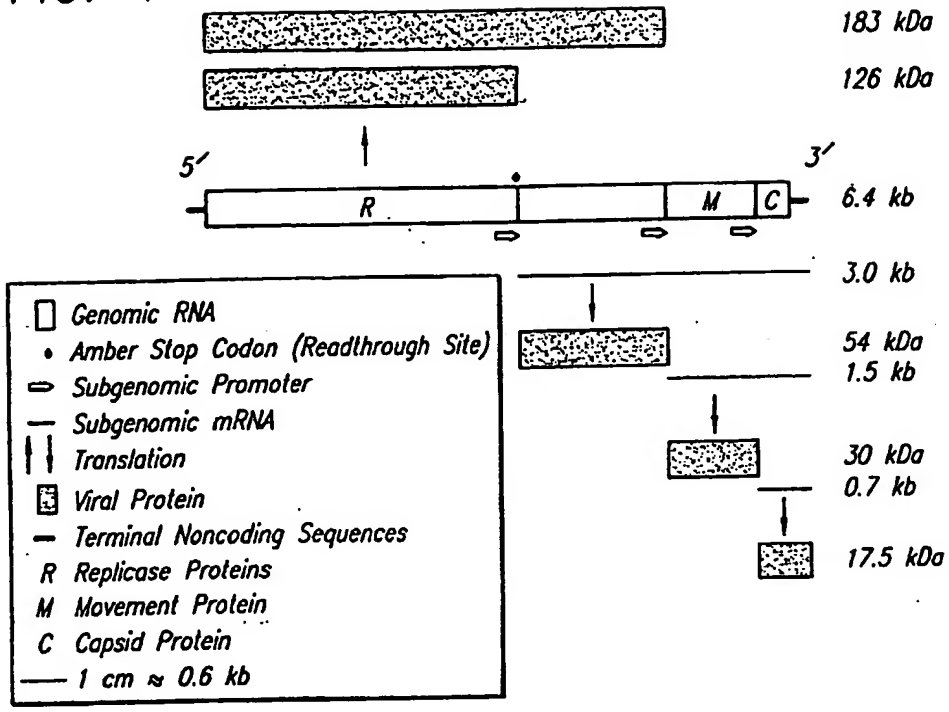

183 kDa
126 kDa
5′
3′
R
M
C
6.4 kb
3.0 kb
54 kDa
1.5 kb
30 kDa
0.7 kb
17.5 kDa
Genomic RNA
Amber Stop Codon (Readthrough Site)
Subgenomic Promoter
Subgenomic mRNA
Translation
Viral Protein
Terminal Noncoding Sequences
R Replicase Proteins
M Movement Protein
C Capsid Protein
1 cm ≈ 0.6 kb

FIG. 2

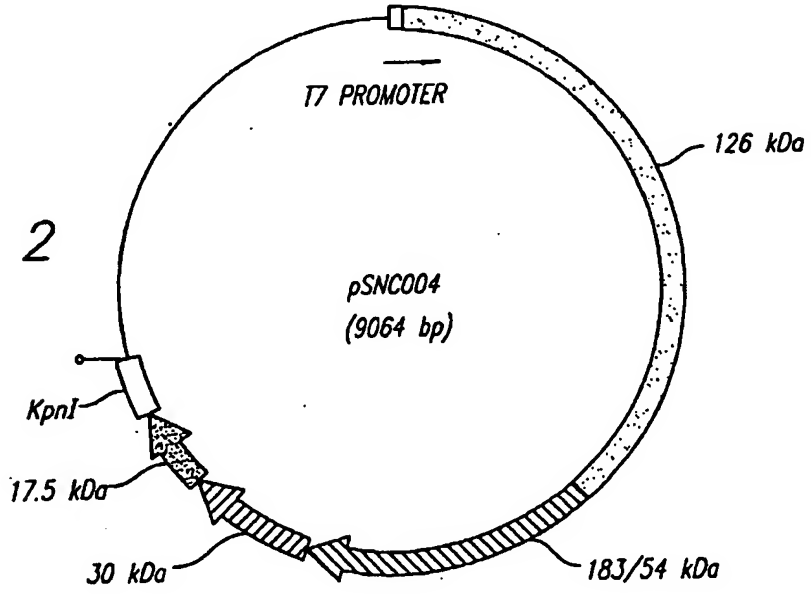

T7 PROMOTER
126 kDa
pSNC004
(9064 bp)
KpnI
17.5 kDa
30 kDa
183/54 kDa

FIG. 3A-1

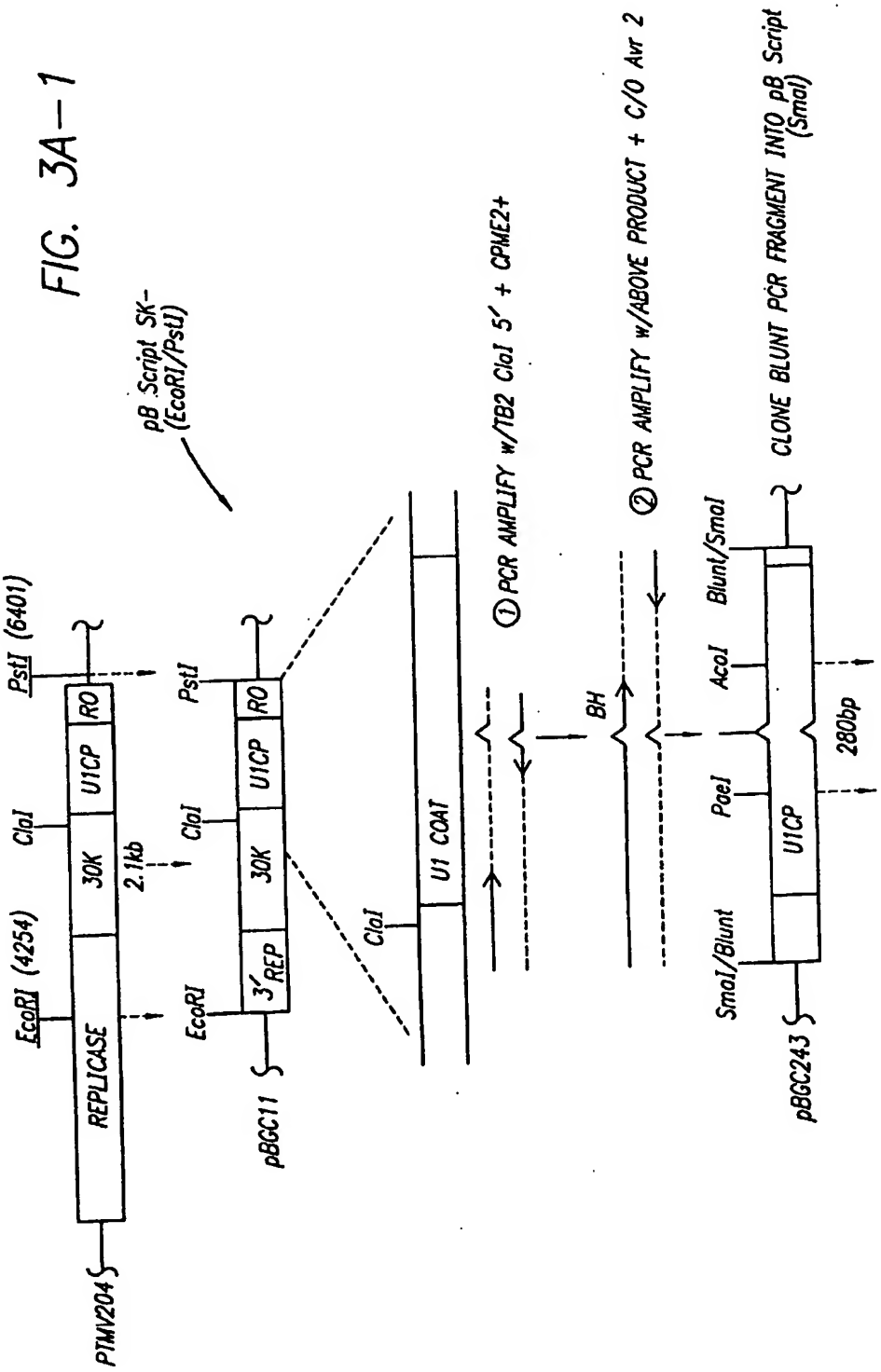
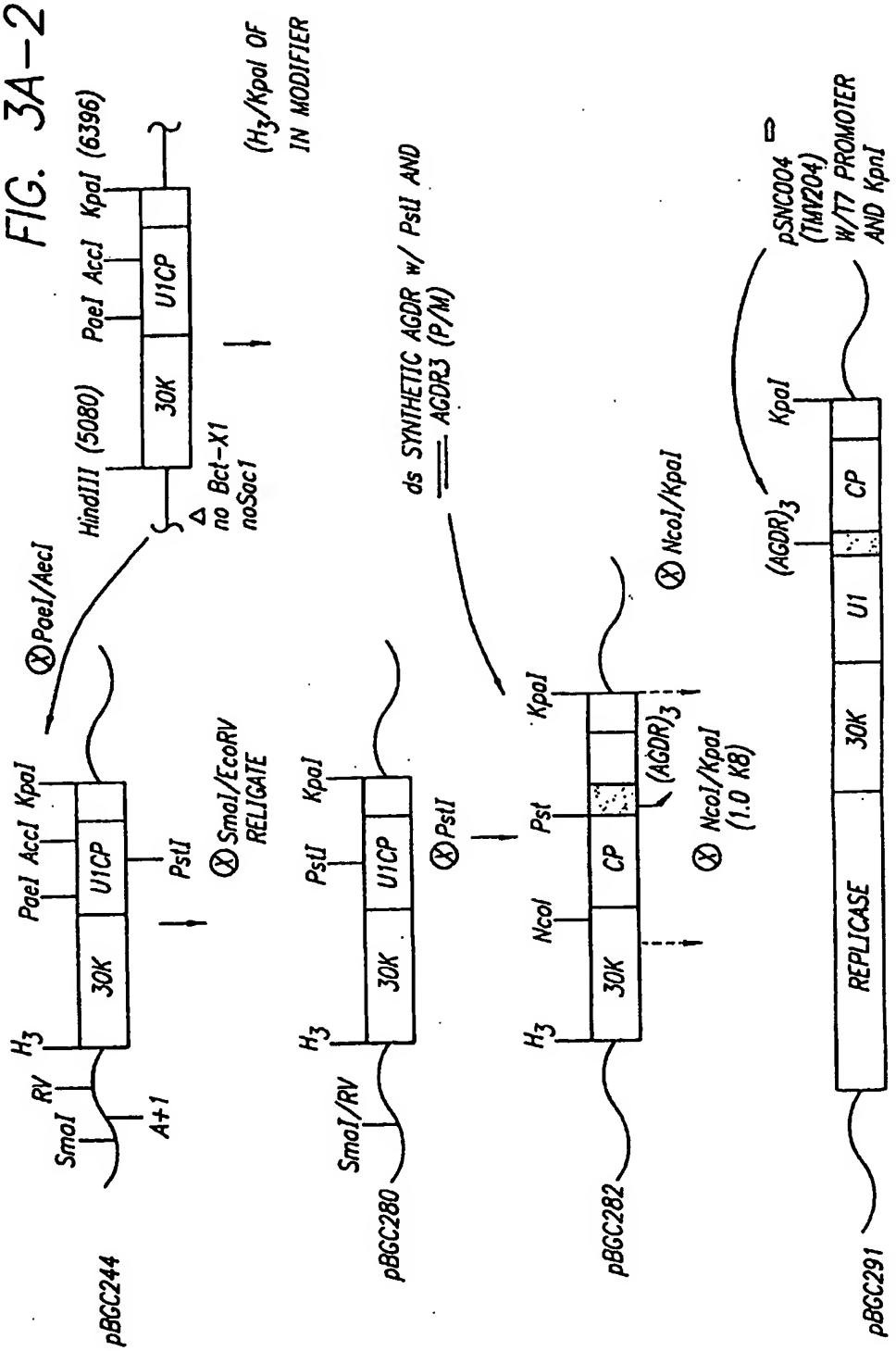

pB Script SK-
(EcoRI/PstI)
PstI (6401)
RO
U1CP
30K
ClaI
2.1kb
EcoRI (4254)
REPLICASE
PTMV204
PstI
RO
U1CP
30K
ClaI
3' REP
EcoRI
pBGC11
ClaI
U1 COAT
① PCR AMPLIFY w/TB2 ClaI 5' + CPME2+
BH
② PCR AMPLIFY w/ABOVE PRODUCT + C/O Avr 2
AcoI
BluntSmaI
PaeI
U1CP
280bp
SmaI/Blunt
pBGC243
CLONE BLUNT PCR FRAGMENT INTO pB Script
(SmaI)

FIG. 3A-2


pBGC244
SmaI
RV
H3
30K
PaeI AccI KpnI
U1CP
PstI
A+1
⊗ SmaI/EcoRV RELIGATE
⊗ PaeI/AecI
HindIII (5080)
PaeI AccI KpnI (6396)
30K
U1CP
Δ no Bct-X1 noSac1
(H3/KpnI OF IN MODIFIER
pBGC280
SmaI/RV
H3
30K
PstI KpnI
U1CP
⊗ PstI
ds SYNTHETIC AGDR w/ PstI AND AGDR3 (P/M)
pBGC282
H3
30K
NcoI
CP
Pst KpnI
(AGDR)3
⊗ NcoI/KpnI
⊗ NcoI/KpnI (1.0 KB)
pBGC291
REPLICASE
30K
U1
CP
(AGDR)3
KpnI
pSNC004 (TMN204) W/T7 PROMOTER AND KpnI

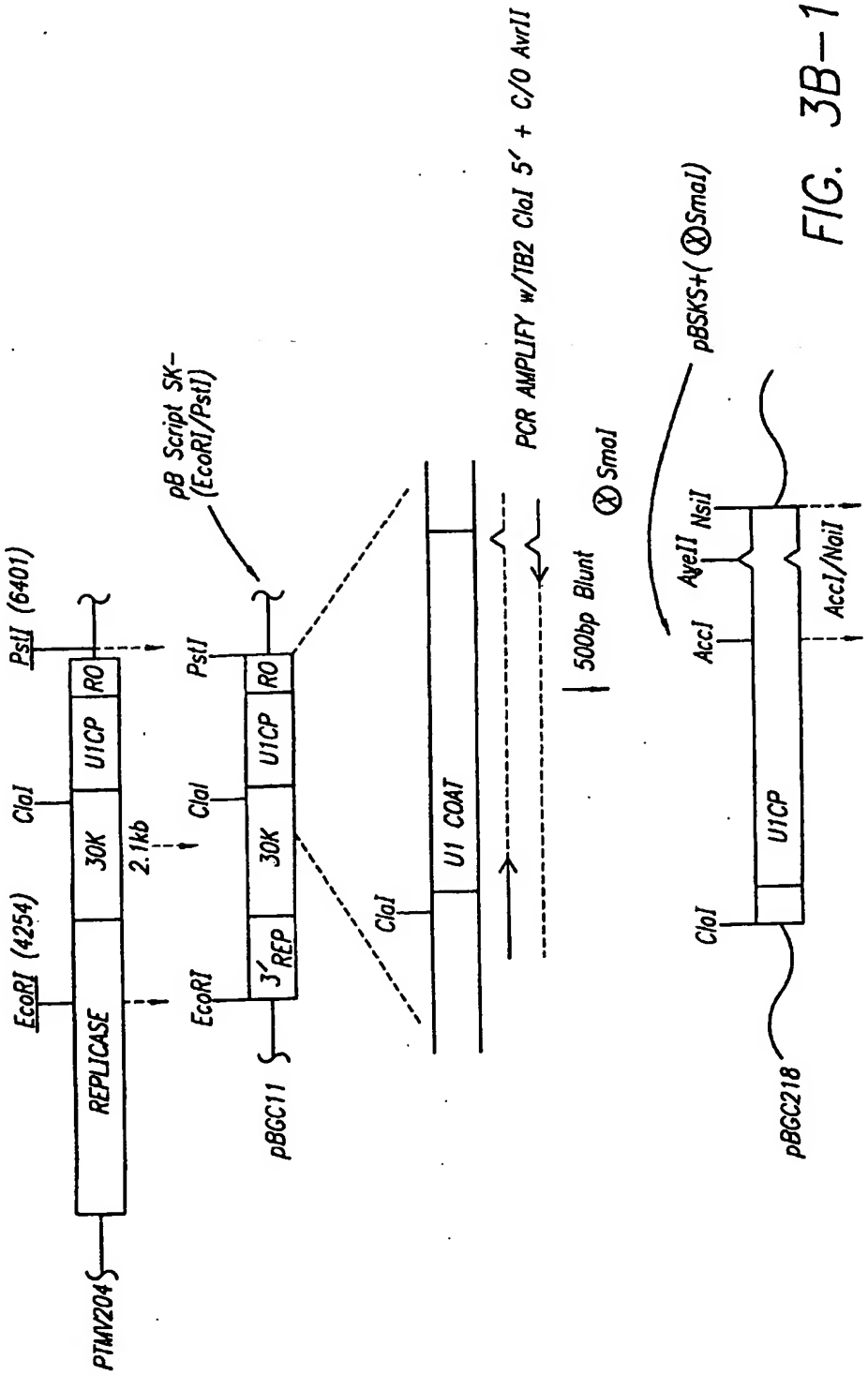

PstI (6401)
RO
U1CP
ClaI
30K
2.1kb
EcoRI (4254)
REPLICASE
PTMV204
pB Script SK-
(EcoRI/PstI)
PstI
RO
U1CP
ClaI
30K
EcoRI
3' REP
pBGC11
ClaI
U1 COAT
PCR AMPLIFY w/TB2 ClaI 5' + C/O AvrII
500bp Blunt
⊗ SmaI
pBSKS+(⊗SmaI)
AccI
AveII
NsiI
AccI/NaiI
ClaI
U1CP
pBGC218

FIG. 3B-1

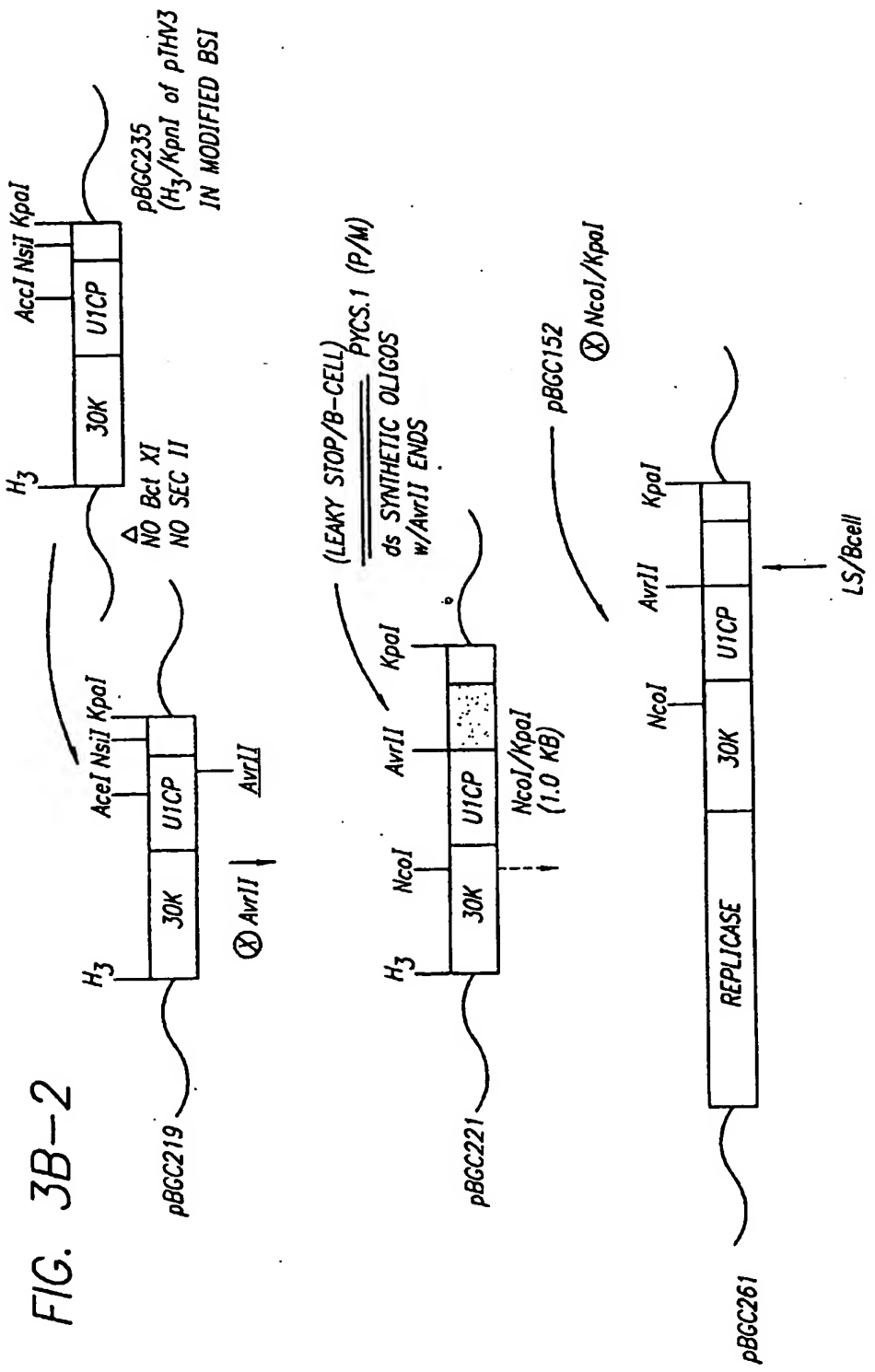

pBGC219
H3
30K
U1CP
AccI NsiI KpnI
AvrII
pBGC235
(H3/KpnI of pTHV3
IN MODIFIED BSI
NO BclI XI
NO SEC II
(LEAKY STOP/B-CELL)
PYCS.1 (P/M)
ds SYNTHETIC OLIGOS
w/AvrII ENDS
pBGC221
NcoI/KpnI
(1.0 KB)
pBGC152
NcoI/KpnI
REPLICASE
LS/BclI
pBGC261

FIG. 3B-2

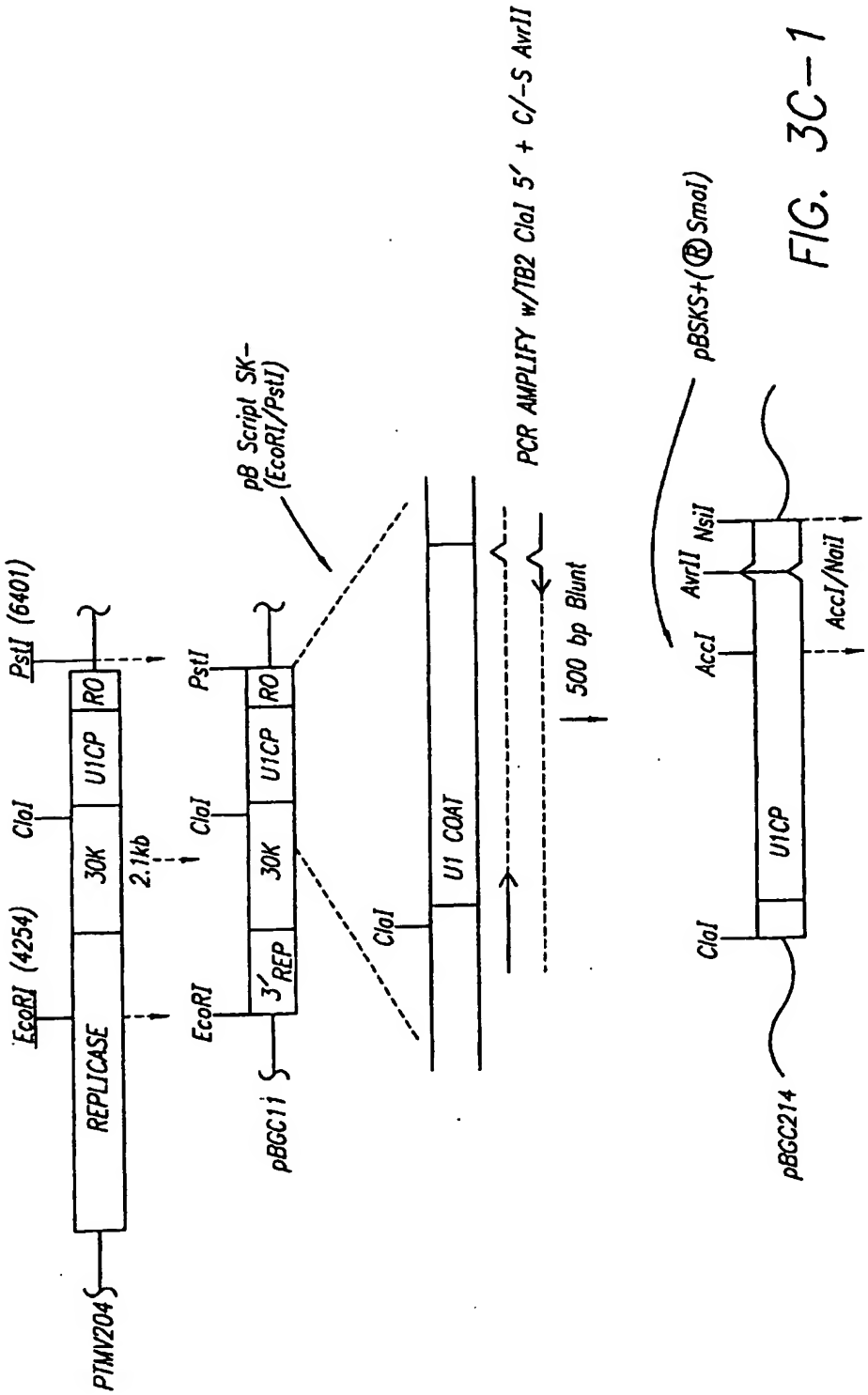
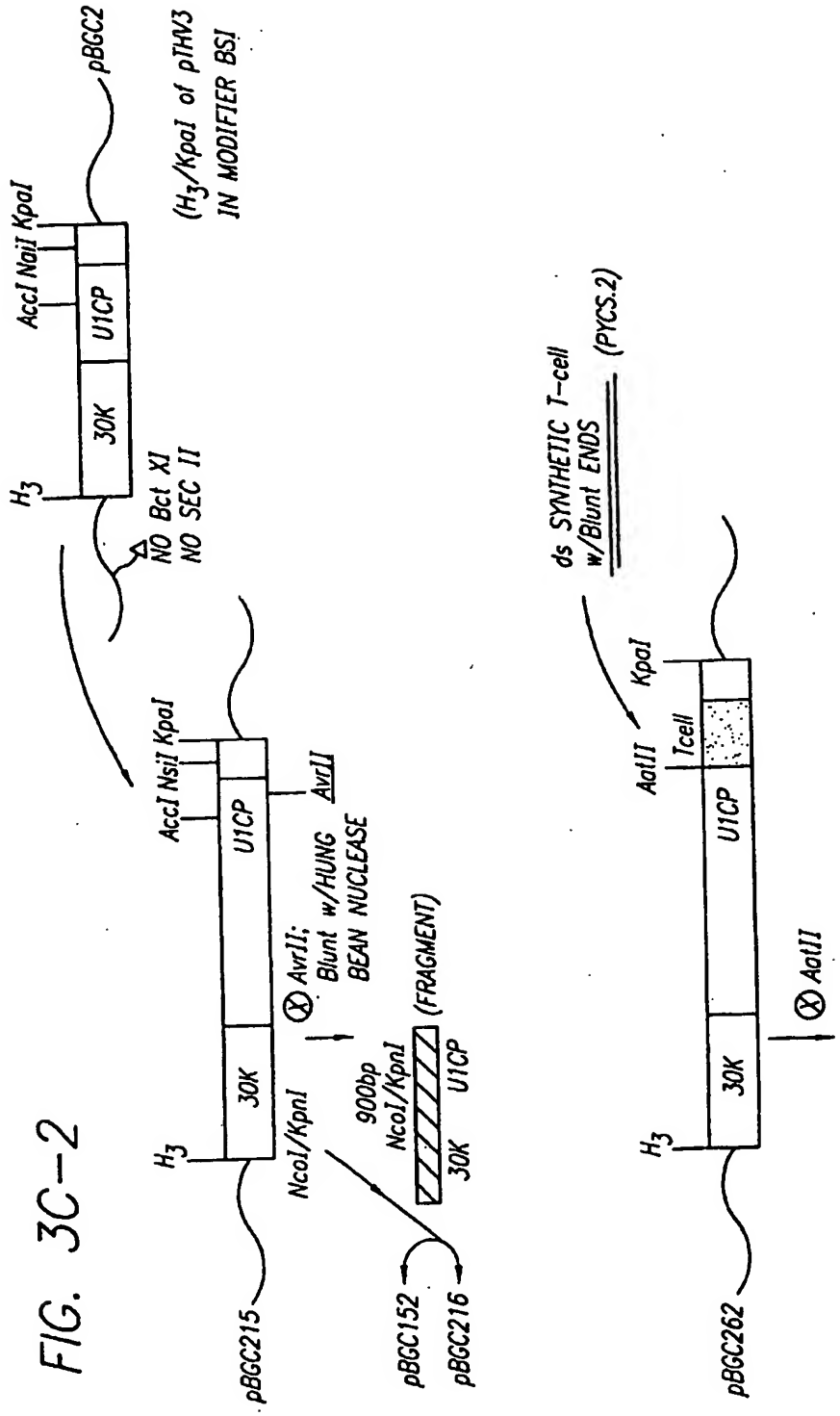

PstI (6401)
RO
U1CP
ClaI
30K
2.1kb
EcoRI (4254)
REPLICASE
PTMV204
pB Scripl SK-
(EcoRI/PstI)
PstI
RO
U1CP
ClaI
30K
EcoRI
3' REP
pBGC11
PCR AMPLIFY w/TB2 ClaI 5' + C/-S AvrII
ClaI
U1 COAT
500 bp Blunt
pBSKS+(® SmaI)
AvrII NsiI
AccI
AccI/NaiI
ClaI
U1CP
pBGC214

FIG. 3C-1

FIG. 3C-2


pBGC2
(H3/KpaI of pTHV3
IN MODIFIER BSI
AccI NaiI KpaI
U1CP
30K
H3
NO BclI XI
NO SEC II
AccI NsiI KpaI
U1CP
AvrII
30K
H3
NcoI/KpnI
⊗ AvrII;
Blunt w/HUNG
BEAN NUCLEASE
900bp
NcoI/KpnI
(FRAGMENT)
30K U1CP
pBGC215
pBGC152
pBGC216
ds SYNTHETIC T-cell
w/Blunt ENDS
(PYCS.2)
KpaI
AatII
Tcell
U1CP
30K
H3
⊗ AatII
pBGC262

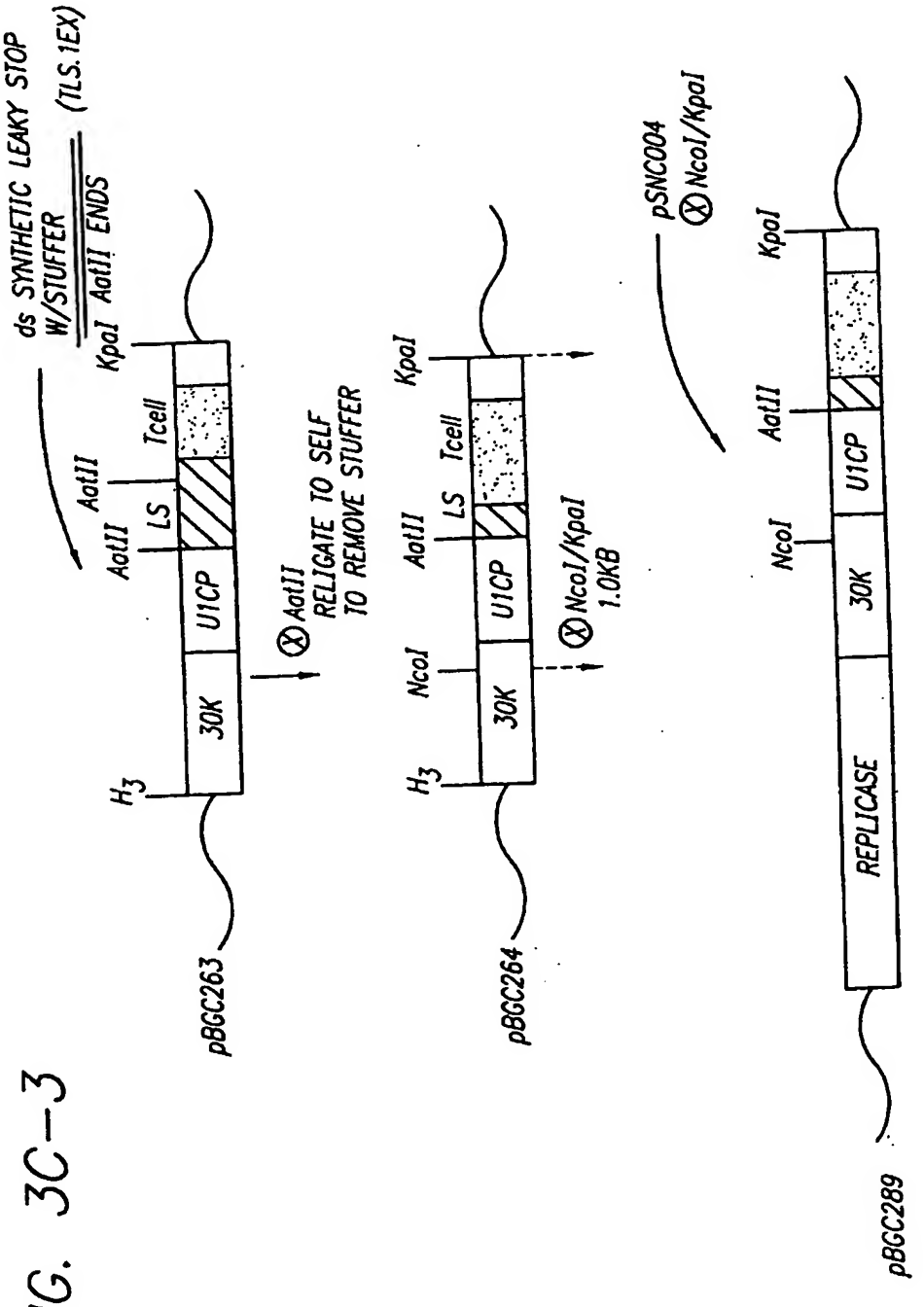

ds SYNTHETIC LEAKY STOP
W/STUFFER (TLS.1EX)
KpaI AatII ENDS
AatII
AatII
LS
TcelI
U1CP
30K
H3
pBGC263
⊗ AatII
RELIGATE TO SELF
TO REMOVE STUFFER
KpaI
AatII
LS
TcelI
NcoI
U1CP
30K
H3
pBGC264
⊗ NcoI/KpaI
1.0KB
pSNC004
⊗ NcoI/KpaI
KpaI
AatII
NcoI
U1CP
30K
REPLICASE
pBGC289

FIG. 3C-3

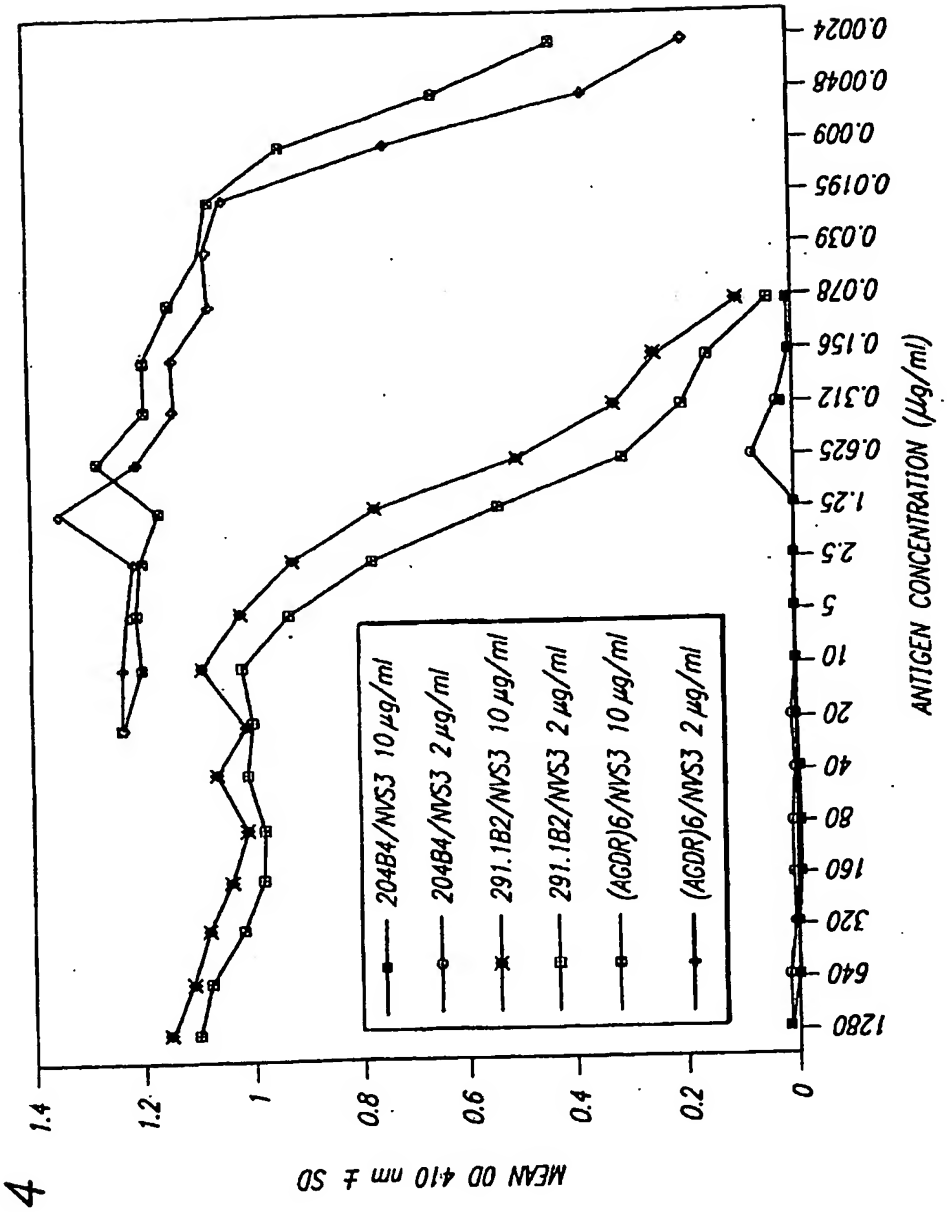

MEAN OD 410 nm ± SD
1.4
1.2
1
0.8
0.6
0.4
0.2
0
ANTIGEN CONCENTRATION (µg/ml)
1280
640
320
160
80
40
20
10
5
2.5
1.25
0.625
0.312
0.156
0.078
0.039
0.0195
0.009
0.0048
0.0024
204B4/NS3 10 µg/ml
204B4/NS3 2 µg/ml
291.1B2/NS3 10 µg/ml
291.1B2/NS3 2 µg/ml
(AGDR)6/NS3 10 µg/ml
(AGDR)6/NS3 2 µg/ml

FIG. 4

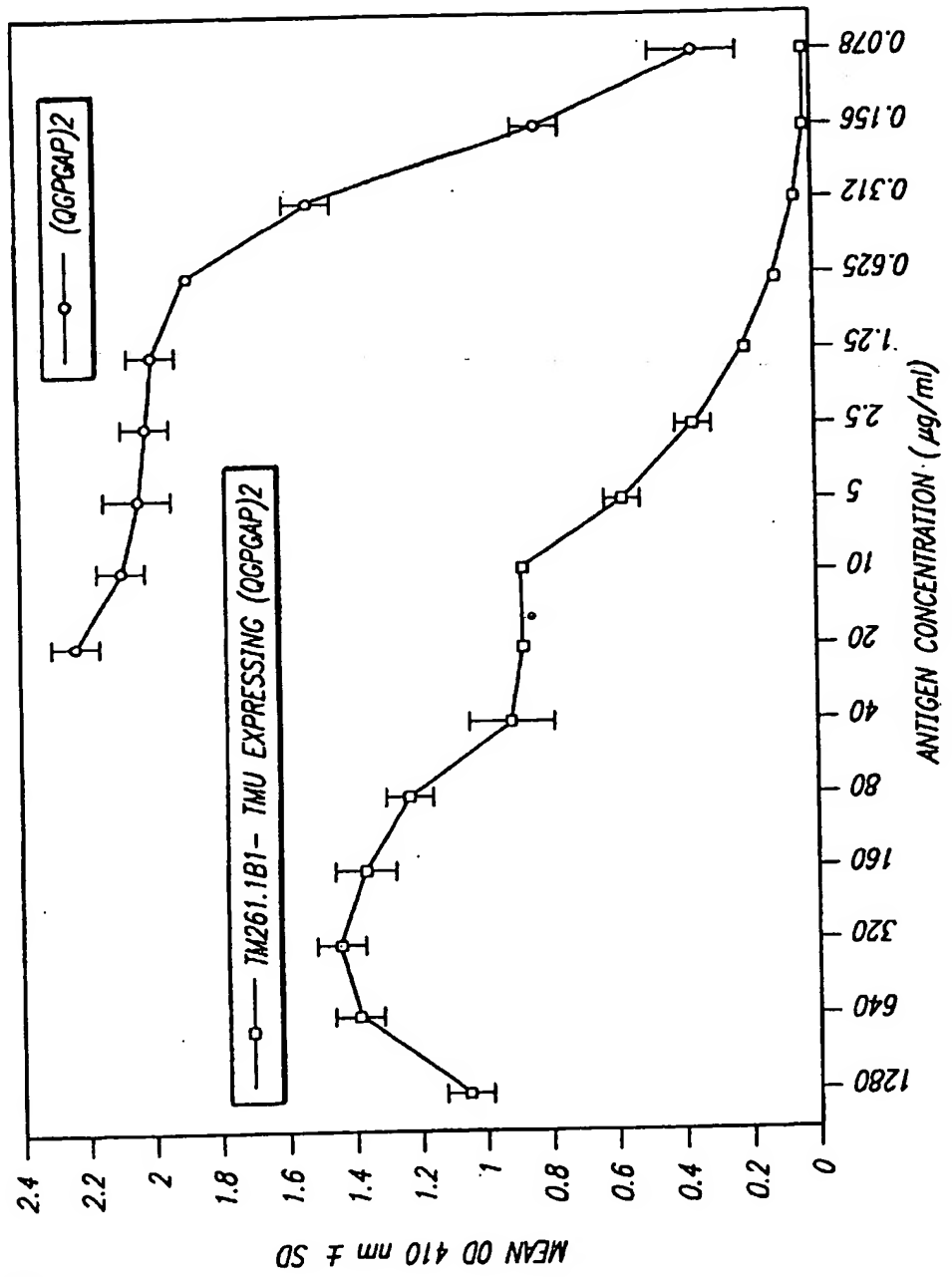
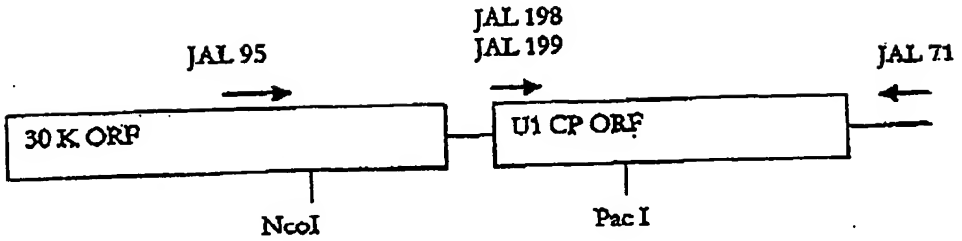
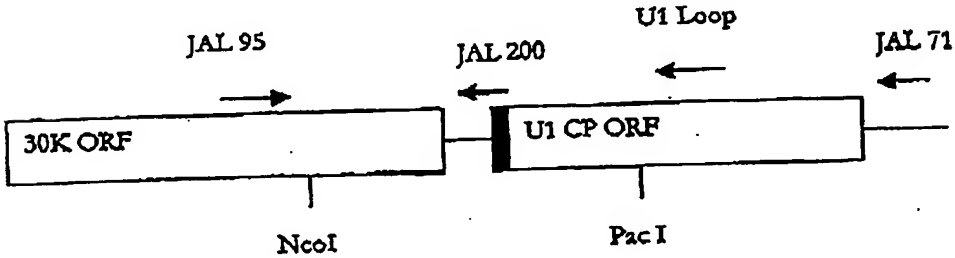

MEAN OD 410 nm ± SD
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
ANTIGEN CONCENTRATION (µg/ml)
1280
640
320
160
80
40
20
10
5
2.5
1.25
0.625
0.312
0.156
0.078
TM261.1B1- TMU EXPRESSING (QGPGAP)2
(QGPGAP)2

FIG. 5

Figure 6



p801



pJL 150

Figure 7
Supernantant 1

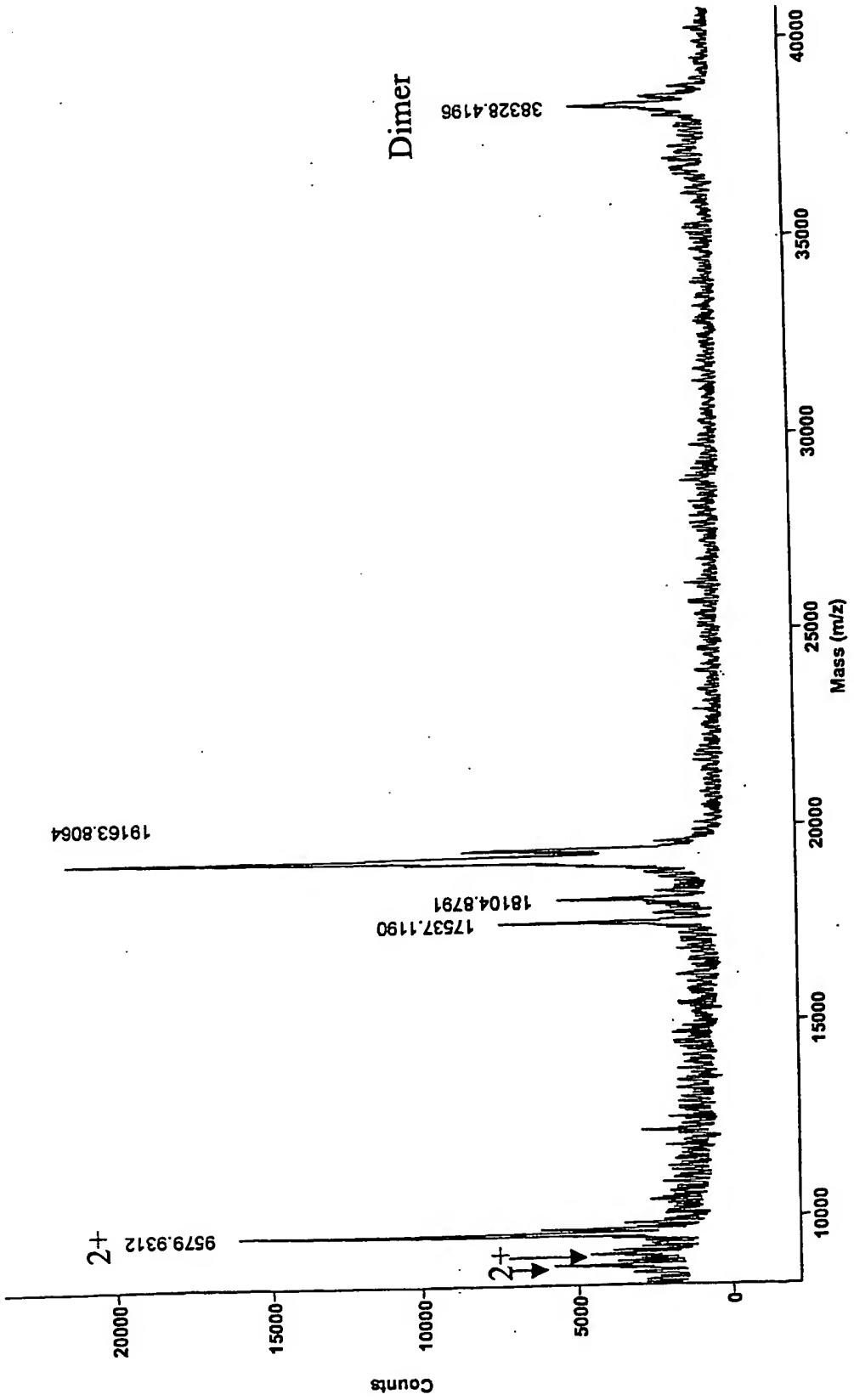


Figure 8
Supernantant 2

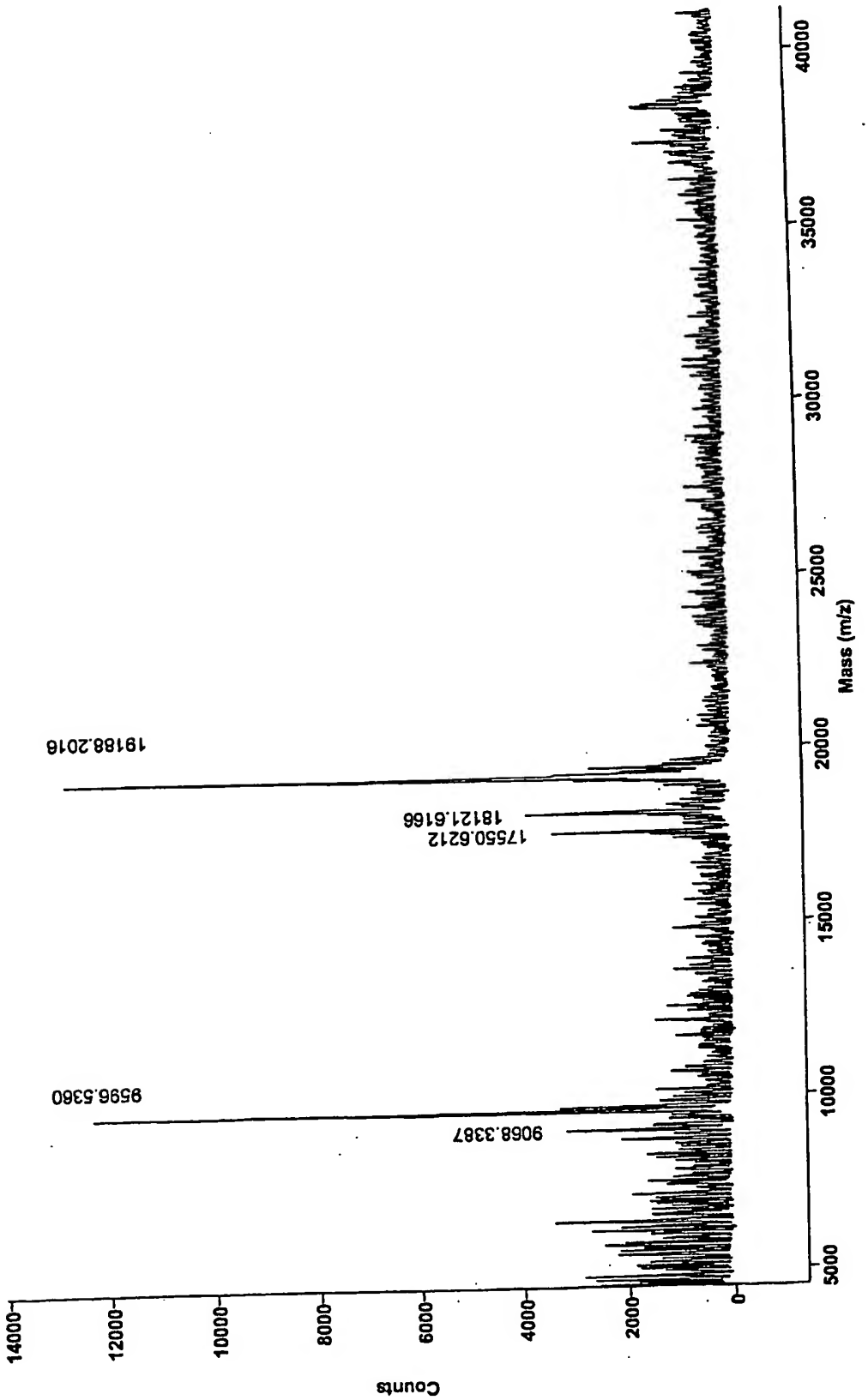


Figure 9

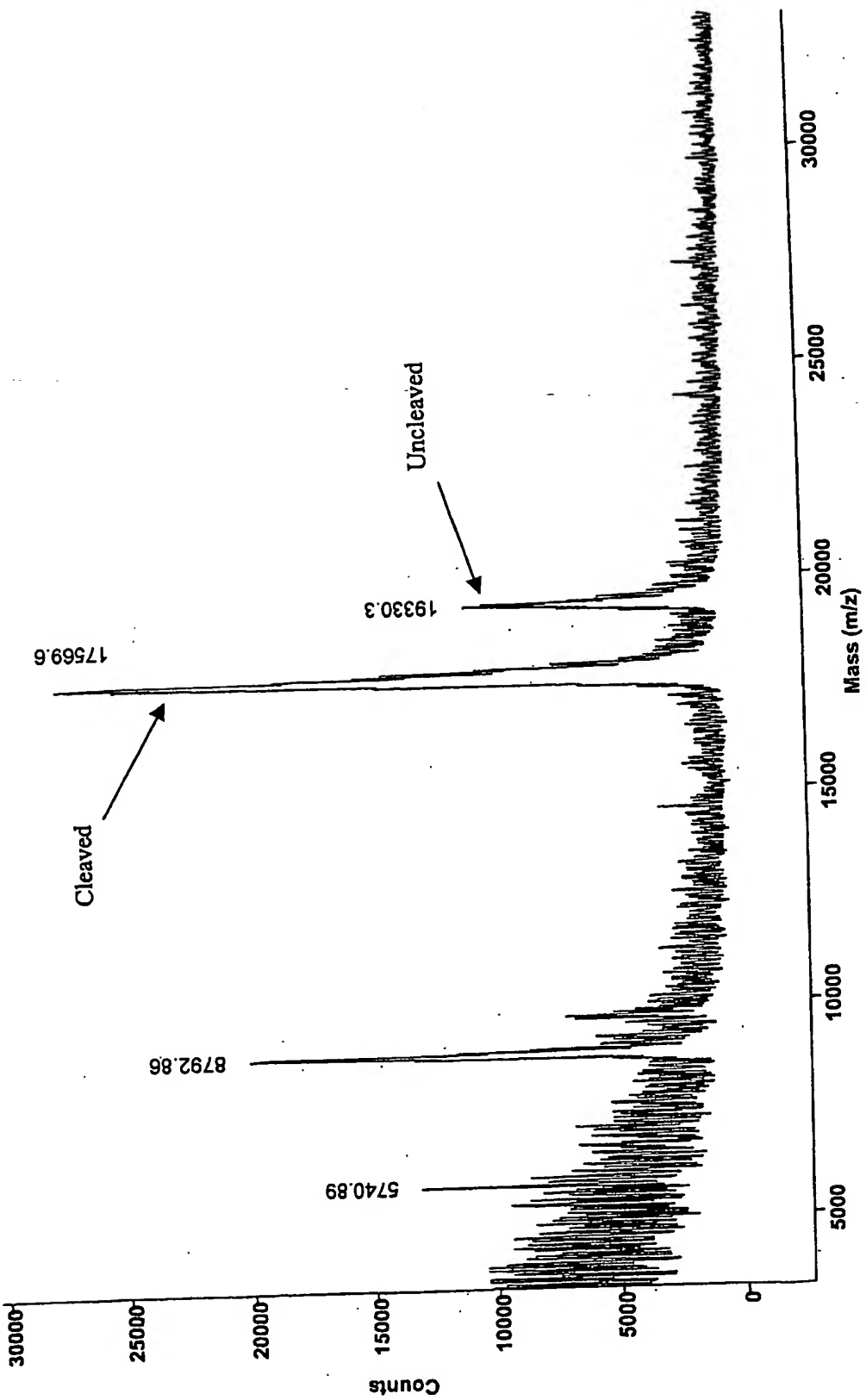

Counts
30000
25000
20000
15000
10000
5000
0
5740.89
8792.86
17569.6
19330.3
Cleaved
Uncleaved
5000
10000
15000
20000
25000
30000
Mass (m/z)

Figure 10

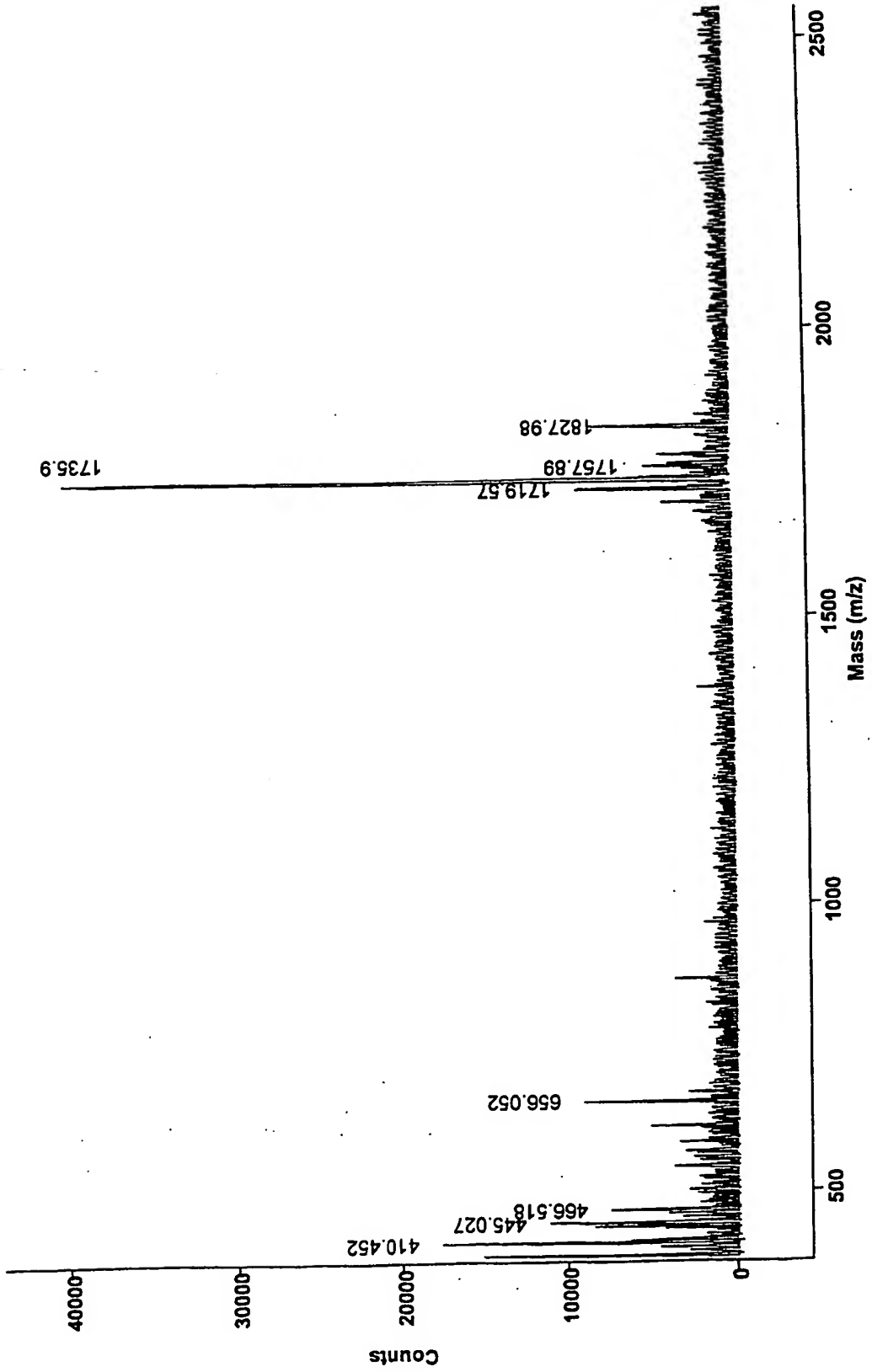

Counts
40000
30000
20000
10000
0
500
1000
1500
2000
2500
Mass (m/z)
410.452
445.027
466.518
656.052
1719.57
1735.9
1757.89
1827.98

Figure 11

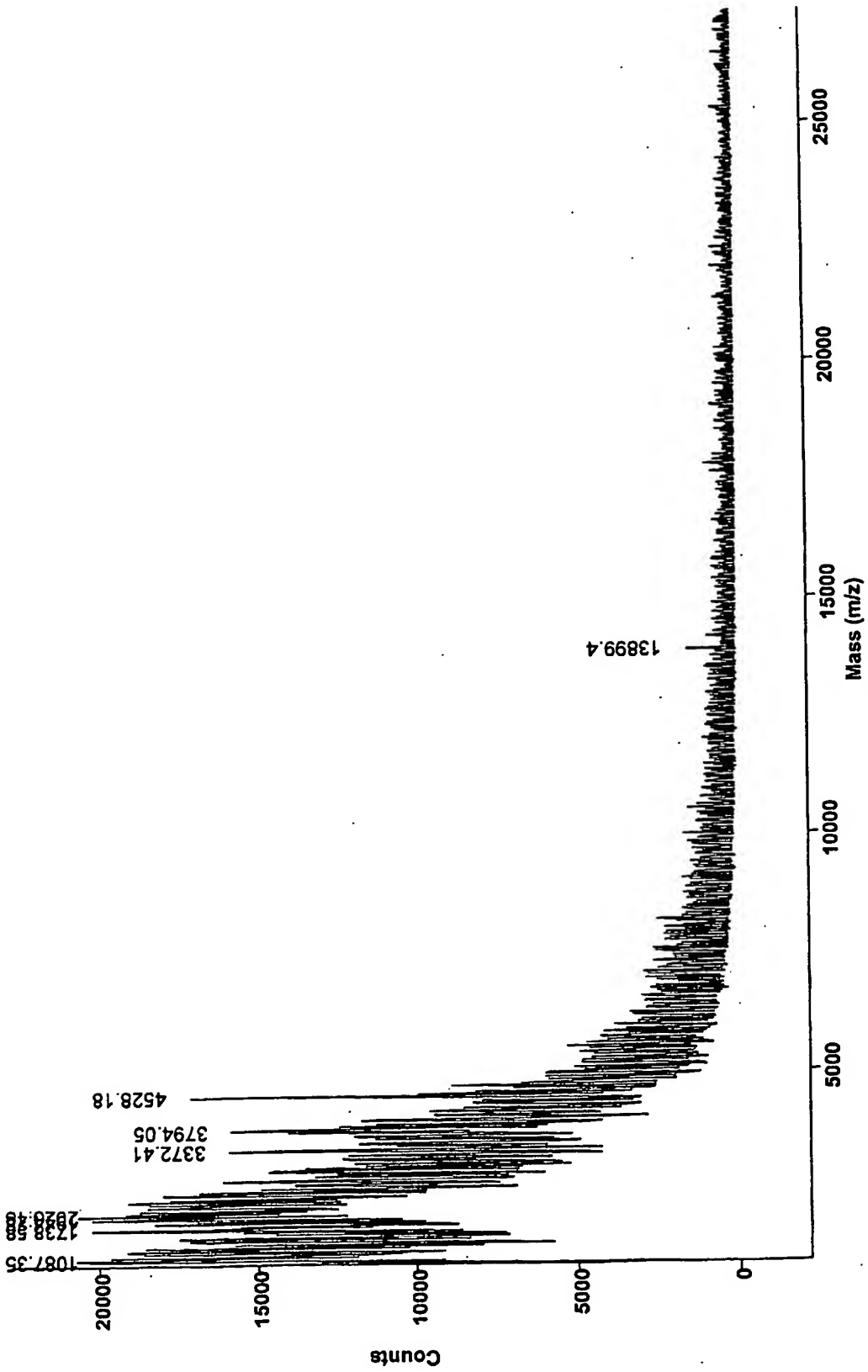

Counts
20000
15000
10000
5000
0
1087.35
1738.58
2020.48
3372.41
3794.05
4528.18
13899.4
5000
10000
15000
20000
25000
Mass (m/z)

Figure 12

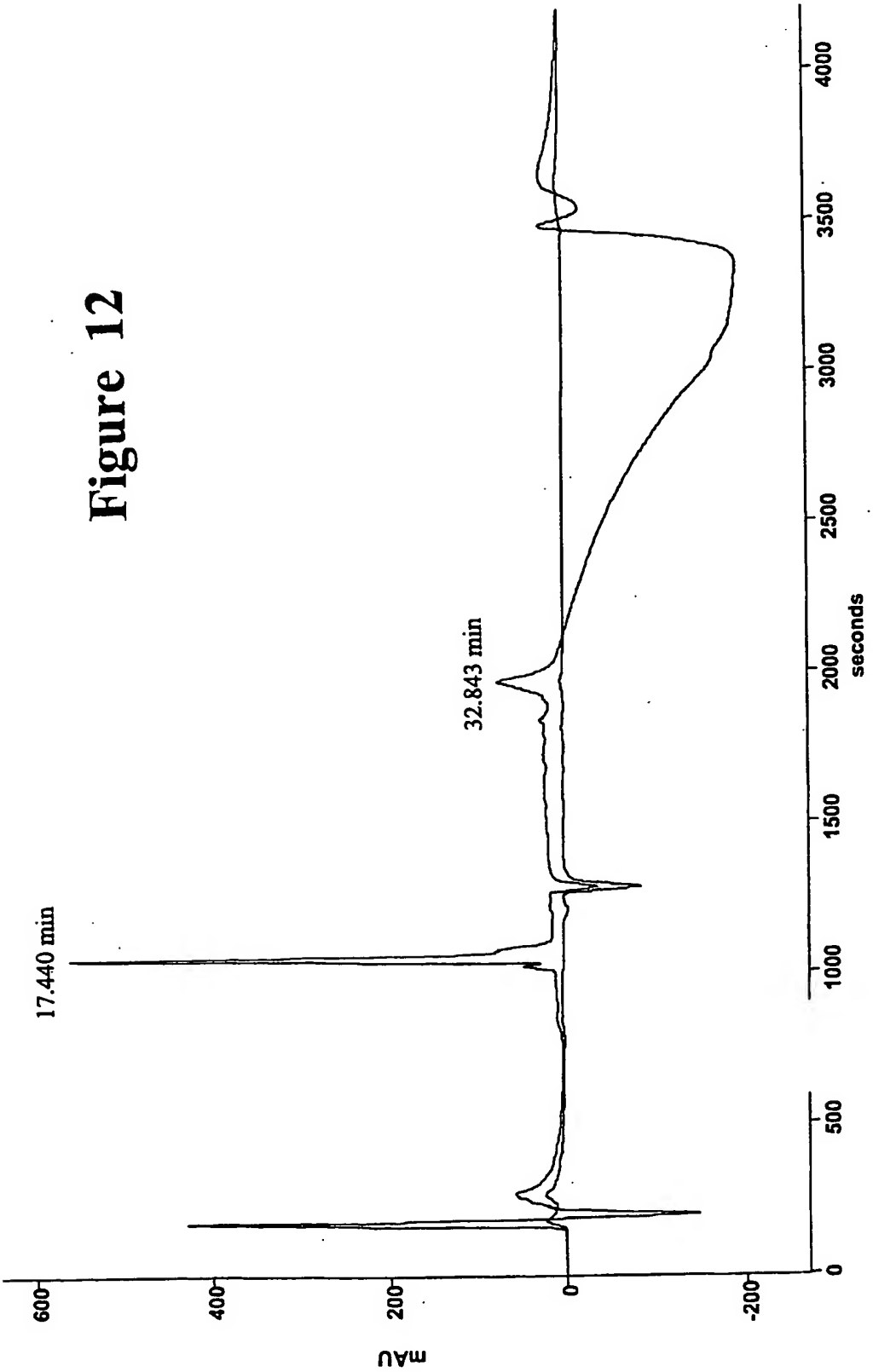

17.440 min
32.843 min
mAU
600
400
200
0
-200
0
500
1000
1500
2000
2500
3000
3500
4000
seconds

Figure 13

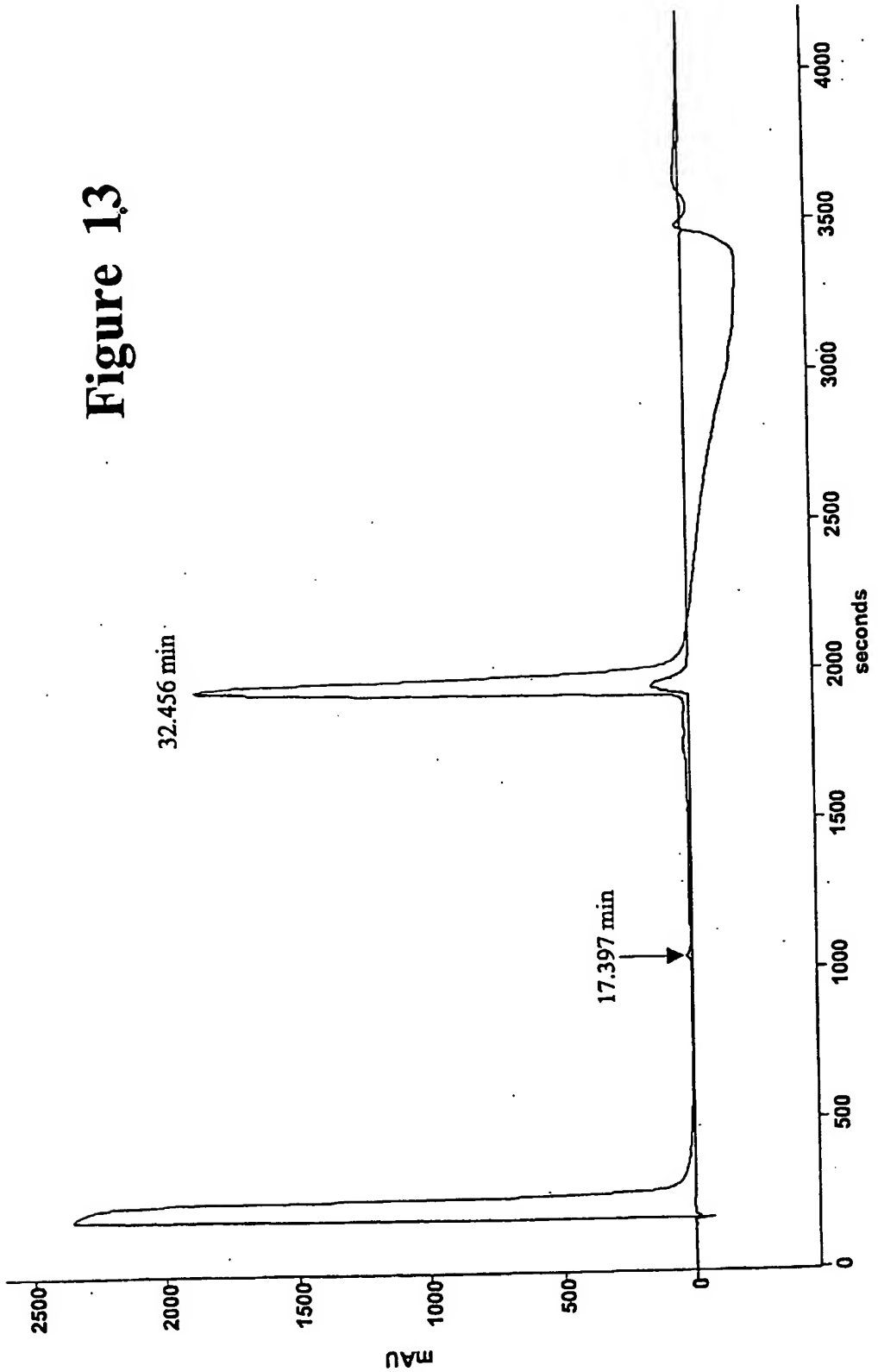

32.456 min
17.397 min
mAU
2500
2000
1500
1000
500
0
0
500
1000
1500
2000
2500
3000
3500
4000
seconds

FIG. 14

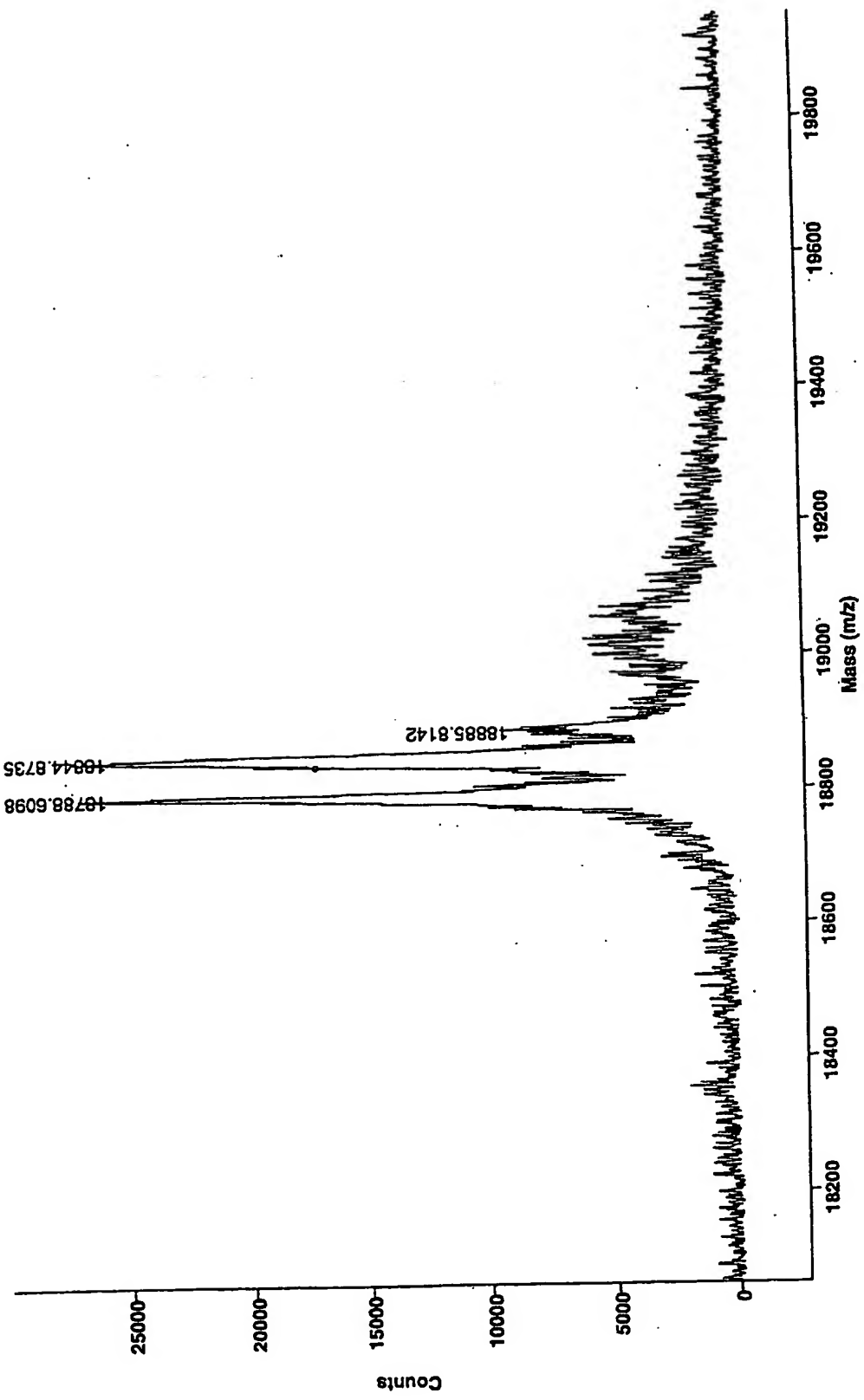

18788.6098
18844.8735
18885.8142
Counts
25000
20000
15000
10000
5000
0
18200
18400
18600
18800
19000
19200
19400
19600
19800
Mass (m/z)

FIG. 15

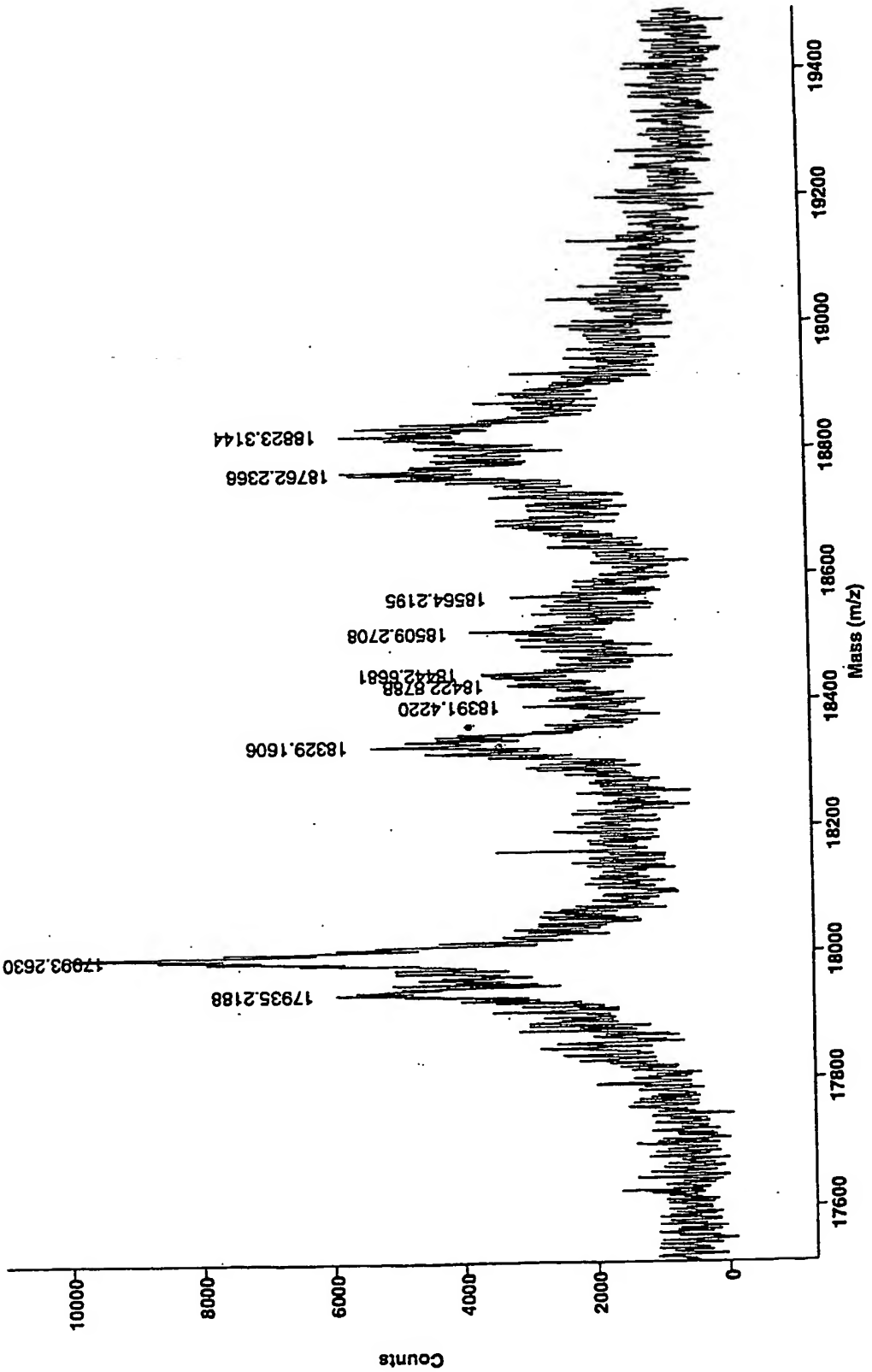

17893.2630
17935.2188
18329.1606
18391.4220
18422.8788
18442.5681
18509.2708
18564.2195
18762.2368
18823.3144
Counts
0
2000
4000
6000
8000
10000
17600
17800
18000
18200
18400
18600
18800
19000
19200
19400
Mass (m/z)

FIG. 16

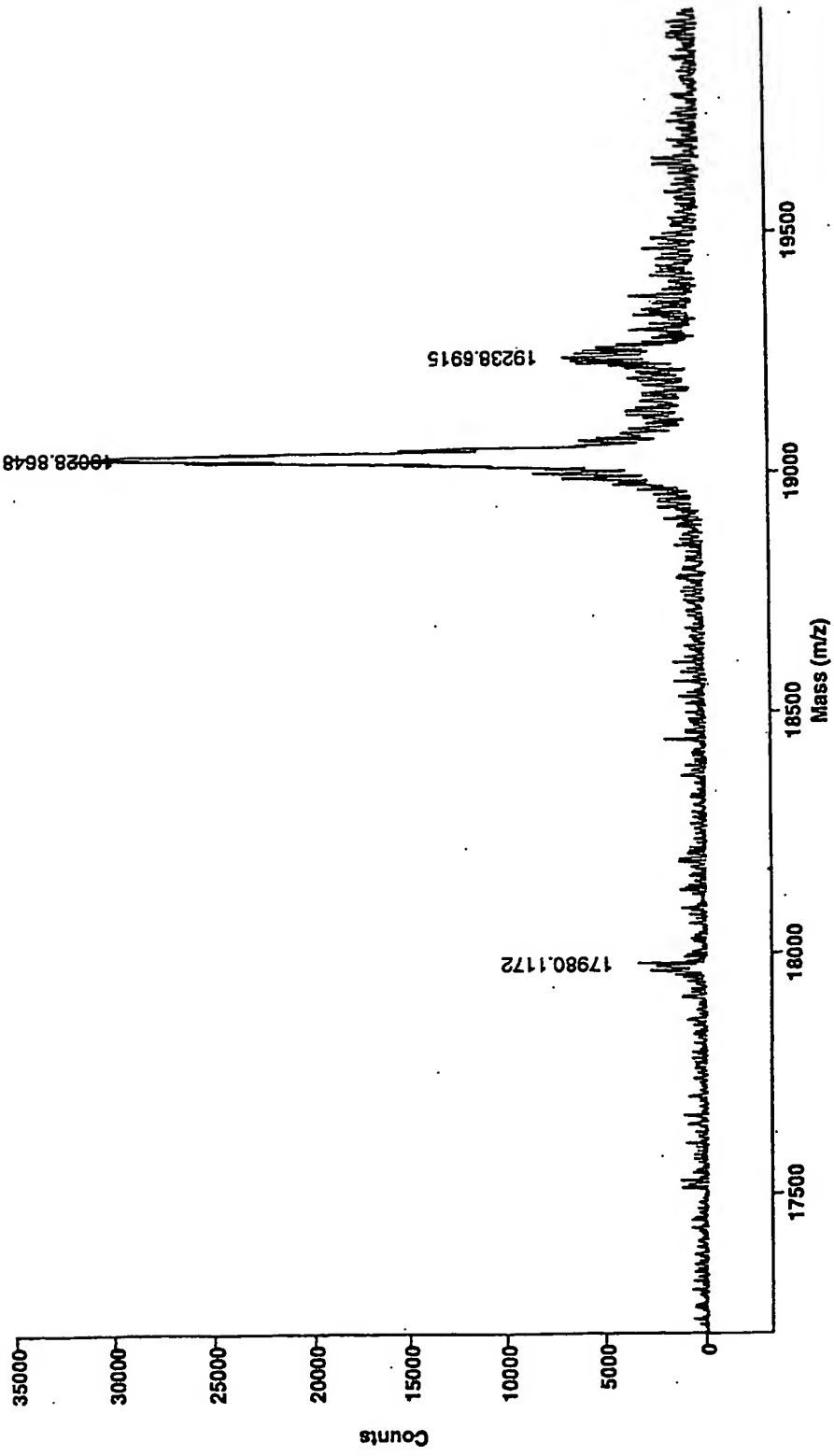

Counts
35000
30000
25000
20000
15000
10000
5000
0
17500
18000
18500
19000
19500
Mass (m/z)
17980.1172
18028.8648
19238.6915

FIG. 17

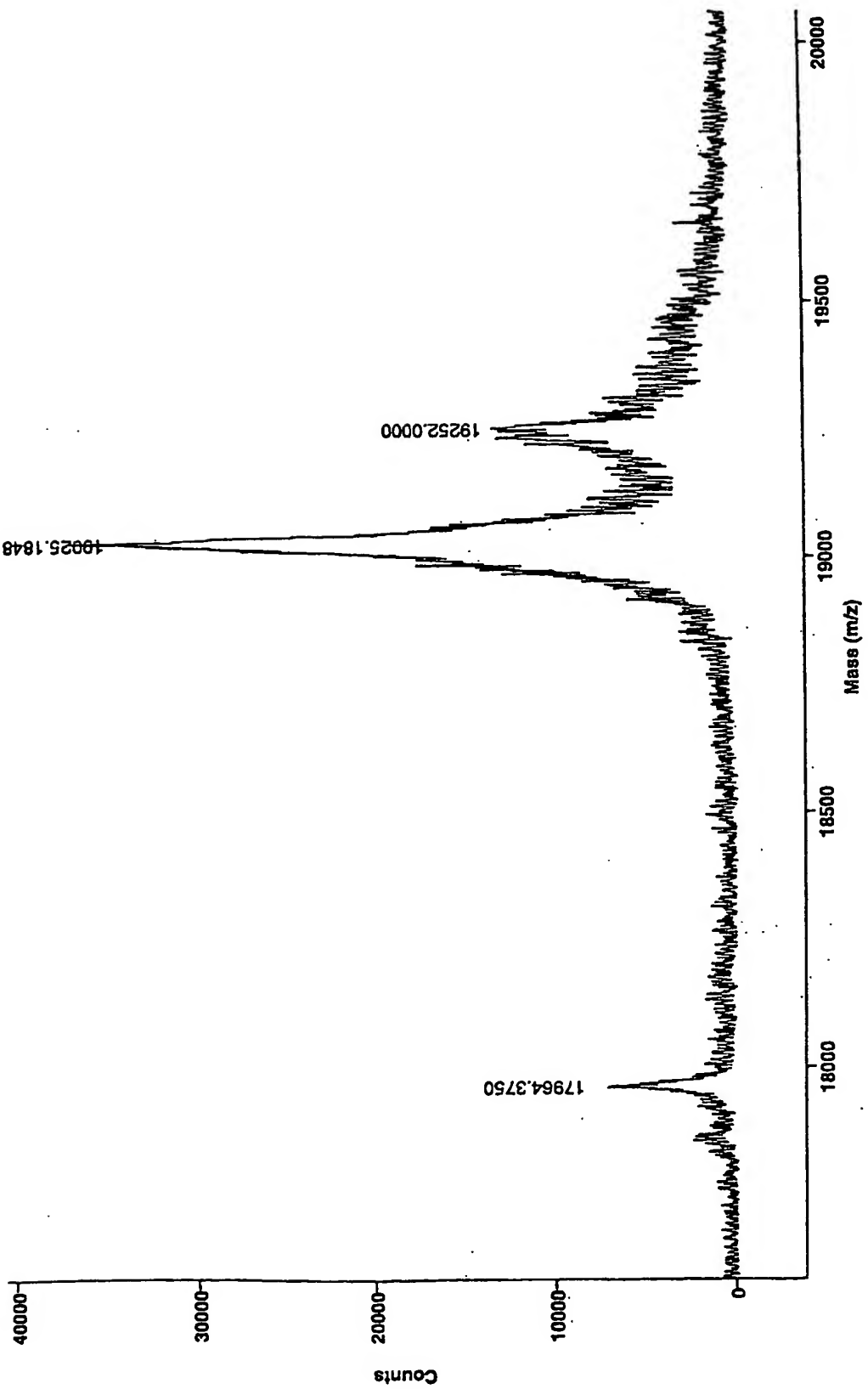

17964.3750
18025.1848
19252.0000
Counts
0
10000
20000
30000
40000
Mass (m/z)
18000
18500
19000
19500
20000

FIG. 18

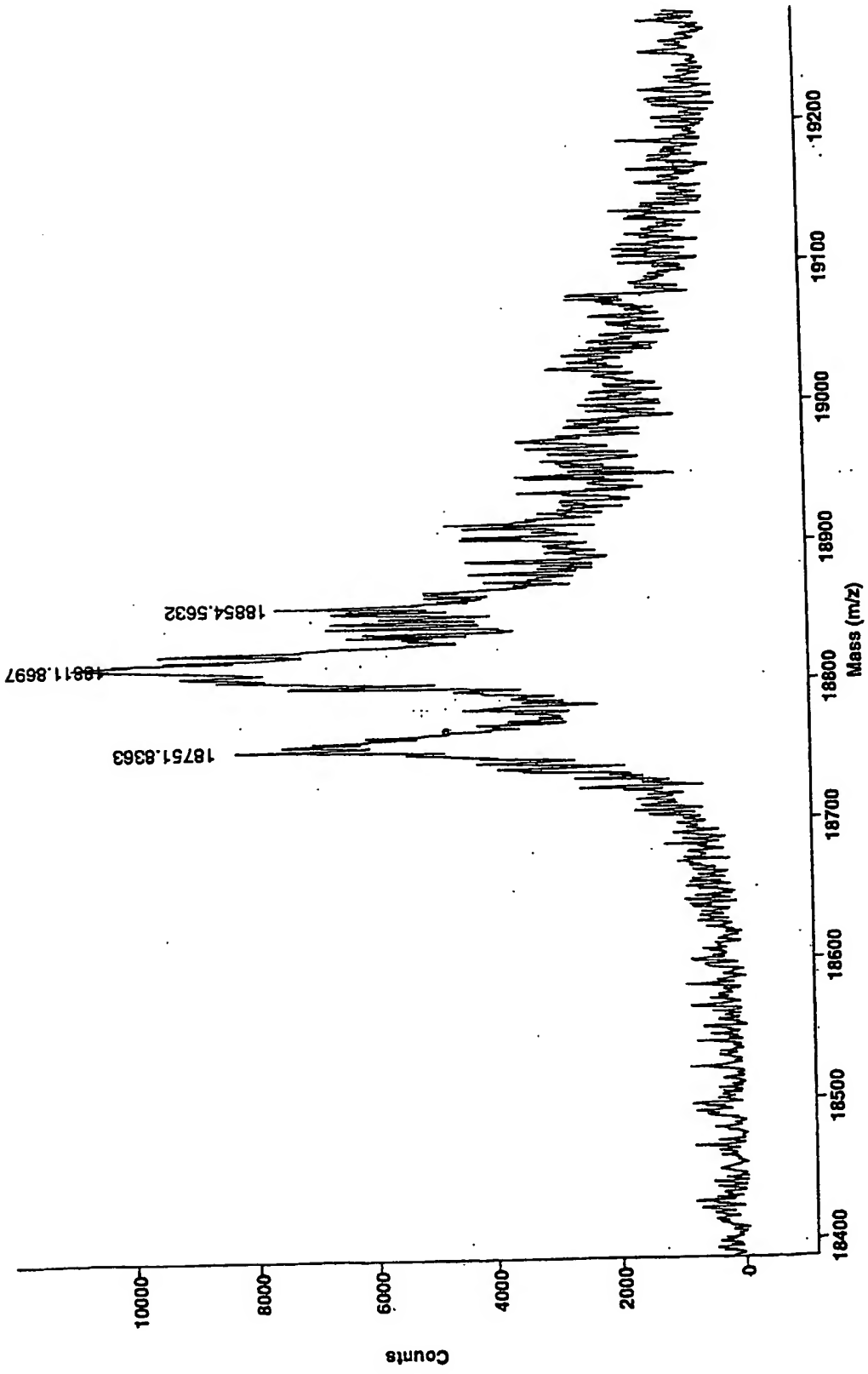
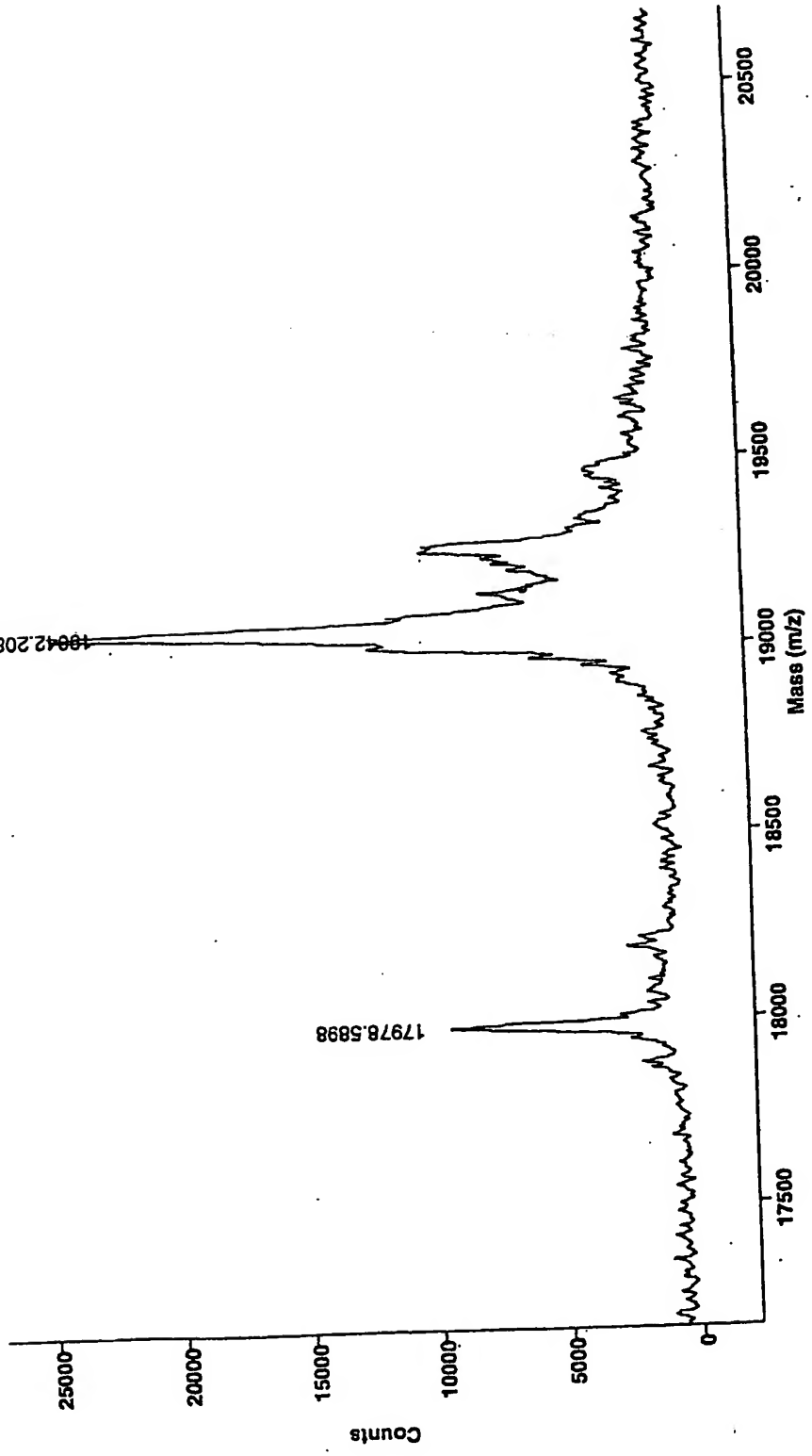

18751.8363
18811.8697
18854.5632
Counts
0
2000
4000
6000
8000
10000
18400
18500
18600
18700
18800
18900
19000
19100
19200
Mass (m/z)

FIG. 19


18042.2083
17978.5898
Counts
25000
20000
15000
10000
5000
0
17500
18000
18500
19000
19500
20000
20500
Mass (m/z)

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